Intersections of animal production practices on meat quality, intramuscular collagen and expression of genes related to collagen and myofibrillar synthesis and degradation

by

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Abstract

Beef quality, particularly tenderness, continues to be a major challenge in the beef industry resulting in significant economic losses. Beef tenderness is influenced by genetic factors, especially the expression of genes associated with collagen and collagen crosslink synthesis, live production factors such as management, including growth promotant (GP) utilization, and *post-mortem* factors such as ageing, processing and packaging. Residual feed intake (RFI) is a measure of feed efficiency and has the potential to increase profitability in the beef industry and reduce green house gas emissions. In this thesis research, our overall aim was to elucidate the effects of RFI and GPs, and their interactions, on growth performance and meat quality of crossbred Angus steers, and the expression of genes associated with collagen and collagen crosslink synthesis. This was achieved by testing different hypotheses in three experimental studies.

A total of 48 crossbred Angus steers classified as low (n = 27) and high (n = 21) RFI status were randomly allocated into steroid (n = 12), beta-agonist (n = 12), combined steroid implant and beta-agonist (n = 12), and control treatments (n = 12). The first study showed an important interaction between RFI and steroid implant to enhance feed conversion efficiency and average daily gain (ADG). The use of steroid implants increased body weight at the end of the treatment phase, although it did not influence the concentration of circulating insulin-like growth factor 1 (IGF-1). Cooked steaks of the *semimembranosus* (SM) muscle from low RFI steers were tougher than those from high RFI steers, as were steaks from both *gluteus medius* (GM) and SM muscles obtained from implanted steers compared to those from non-implanted steers. *Post-mortem* ageing was found to have the most significant effect on meat quality characteristics, particularly on tenderness as steaks from both muscles aged for 12 days were more tender compared to steaks aged for 3 days.

In the second study, we explored the effect of RFI and GPs on the total collagen content of both SM and GM muscles, and the density of two trivalent crosslinks, Ehrlich's chromogen (EC) and pyridinoline (PYR), of the SM muscle. In the SM muscle, insoluble collagen content was influenced by an interaction between RFI, steroid implant and ageing period where it was higher in muscles from steers of low RFI status that were implanted and aged for 3 days than in muscles aged for 12 days. Insoluble collagen content in the GM muscle was lower in muscles from implanted steers than in non-implanted steers. Percentage solubility in the SM muscle increased in muscles from low RFI steers non-supplemented with beta-agonist and aged for 12 days rather than 3 days, while percentage solubility in the GM muscle was higher in muscles aged for 12 days than for 3 days. The intramuscular connective tissue (IMCT) structure was found to be weakened with *post-mortem* ageing as soluble collagen concentration increased by day 12 in both muscles. While beta-agonist increased the density of both mature crosslinks in the SM muscle, steroid implants decreased EC density but increased PYR density.

The expression of 31 genes associated with collagen and collagen crosslink synthesis, and myofibril degradation were profiled in the third study using quantitative real time polymerase chain reaction. Results showed a lower expression level of *CAPNI* in muscles from low RFI steers treated with steroid implants and ractopamine hydrochloride (RH), than high RFI steers treated with both GPs. The expression of the *CAST* gene was tentatively higher in muscles from low RFI steers, hence the toughness of steaks of SM muscles from low RFI steers, supported by the tendency for *MMP13* to have low expression in SM muscles from low RFI steers. Steroid implant did not increase the expression levels of collagen synthesising genes *COL1A1*, *COL3A1, ITGA11, ITGB1, TIMP1, TIMP2,* and *FN1.* Results from this PhD research thesis contribute to knowledge on selection for improved RFI and its effect on live production and meat quality characteristics,

and the advantages and disadvantages of the use of GPs individually and in combination on live production and meat quality characteristics, and on the expression of target genes.

Preface

Challenges with meat quality, particularly inconsistency in beef tenderness continue to plague the beef industry, causing economic losses. The different breeds of cattle used by producers, combined with different production and management systems result in many different factors that influence beef tenderness. Due to the demand of high quality, and particularly consistent tender beef by consumers, it was imperative to elucidate the underlying factors, physiological as well as those that affect gene expression, in a bid to combat beef quality inconsistency. This thesis therefore incorporates a number of different but related studies to address various hypotheses.

For Chapter 2, growth performance data was collected by periodic visits to the Roy Berg Kinsella Research Ranch in Vegreville, Alberta. Beef samples were collected from a provincially inspected commercial abattoir where cattle were slaughtered in compliance with the Canadian Council Animal Care Guidelines. I actively participated in the entire sampling and laboratory procedures. Dr. Bimol Roy and the members of Dr. Bruce's laboratory assisted in performing some of the meat quality analyses at the Agri-Food Discovery Place. I performed the Warner-Bratzler Shear Force analysis at the Agri-Food Discovery Place, and proximate analysis at the Meat Biochemistry Laboratory of the Department of Agriculture, Food, and Nutritional Science at the University of Alberta. This chapter has been written into a manuscript and submitted to the Translational Journal of Animal Science. Dr. Bimol Roy and Dr. Heather Bruce are the co-authors.

For muscle collagen concentration and collagen crosslink density (Chapter 3), samples collected during meat quality analysis, frozen and stored at -20 ℃ were used for this analysis. The details of the sample collection, and experimental procedure which I performed at the Meat Biochemistry Laboratory of the Department of Agriculture, Food, and Nutritional Science at the University of Alberta, are presented in Chapter 3. For the measure of the density of the crosslink pyridinoline, final samples were sent for quantification by high pressure liquid chromatography at the Department of Agriculture, Food, and Nutritional Science at the University of Alberta. I performed all the statistical analysis and their interpretation, with assistance from Dr. Heather Bruce.

For muscle gene expression (chapter 4), the samples analyzed were collected from the provincial beef abattoir. The details of the sample collection and experimental procedures are detailed in chapter 4. Dr. Leluo Guan provided guidance, and Yanhong Chen assisted in conducting the experiment at the Agricultural Genomics and Proteomics Unit of the Department of Agriculture,

Food, and Nutritional Science at the University of Alberta. I performed all the statistical analyses and their interpretation, with assistance from Dr. Heather Bruce.

Patience Coleman

Dedication

To God almighty, whose grace has been sufficient for me

Acknowledgement

I am highly grateful to Dr. Heather Lee Bruce for her supervision and support that enabled me to undertake and complete my PhD programme. I deeply appreciate the opportunity she gave me to enhance my knowledge, and also for her transfer of knowledge in Meat Science. Your support, encouragement and teachings urged me on to improve myself, and also form a sound foundation in research as I embark on my career journey as a Meat Scientist. I am grateful to Drs. Michael Steele and Leluo Guan for their support and guidance as members of my committee. I appreciate your kindness, thought-provoking mentoring and constructive criticism. I am grateful to Drs. Michael Dyck and Frank Robinson for their knowledge contribution and for being external examiners during my candidacy exam.

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Table of Contents

List of Tables

List of Figures

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
β-AA	Beta-adrenergic agonists
BW	Body weight
cAMP	Cyclic-adenosine monophosphate
CCA	Canadian Cattlemen's Association
CCAC	Canadian Council of Animal Care
cDNA	Complementary deoxyribonucleic acid
CSA	Cross-sectional area
Ct	Cycle threshold
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOA	Days of ageing
dpm	Days post-mortem
EC	Ehrlich's chromogen
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISA	American Science Association
ER	Endoplasmic reticulum
FAS/USDA	Foreign Agricultural Service/United States Department of Agriculture
FCR	Feed conversion ratio
FE	Feed efficiency
FGF2	Fibroblast growth factor-2
G:F	Gain to feed ratio
GDP	Gross domestic product
GGT	Galactosylhydroxylysyl glucosyltransferase
GLM	General linear model
GM	Gluteus medius
GP	Growth promotant
GT	Galactosyltransferase
HCl	Hydrochloric acid
HCW	Hot carcass weight
HFBA	Heptafluorobutyric acid
HK	Housekeeping
HP	Hydroxylysyl pyridinoline
HPLC	High performance liquid chromatography
HSD	Honestly Significant Difference
IGF-1	Insulin-like growth factor 1

IGFBP-3	Insulin-like growth binding protein 3
IMCT	Intramuscular connective tissue
IMF	Intramuscular fat
IMP	Implant
KR	Kleiber ratio
LD	Longissimus dorsi
LH	Lysyl hydroxylase
LOX	Lysyl oxidase
LT	Longissimus thoracis
MEaTnet	Meat Education and Training Network
MFI	Myofibrillar fragmentation indices
MMLV	Moloney murine leukemia virus
MMPs	Matrix metalloproteinases
mRNA	Messenger Ribonucleic Acid
MWT	Metabolic body weight
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NSERC	Natural Sciences and Engineering Research Council
O.D	Optical densities
OMb	Oxymyoglobin
P4H	Prolyl 4-hydroxylases
PhD	Doctor of Philosophy
PKA	Protein kinase A
PP	Pectoralis profundus
PYR	Pyridinoline
qPCR	Quantitative Polymerase Chain Reaction
RFI	Residual feed intake
RFIf	Residual feed intake adjusted for back fat thickness
RGR	Relative growth rate
RH	Ractopamine hydrochloride
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RT-PCR	Real-time Polymerase Chain Reaction
RT-qPCR	Real-time Quantitative Polymerase Chain Reaction
SM	Semimembranosus
TB	Triceps brachii
TBA/E2	Trenbolone acetate/estradiol
TGF-β1	Transforming growth factor beta 1
TIMP1	Tissue inhibitors of metalloproteinase 1
TIMP2	Tissue inhibitors of metalloproteinase 2
TIMPs	Tissue inhibitors of metalloproteinases
USA	United States of America
UV	Ultra-violet
WBSF	Warner-Bratzler Shear Force
WHO	World Health Organization
ZH	Zilpaterol hydrochloride

Chapter 1. General Introduction

Feed constitutes the major cost of input, comprising up to 70% of total production cost (Cattle Fax, 2017). Enhancing the efficiency of feed utilization will aid in the reduction of production cost. Improving feed efficiency of beef animals has been identified and implemented as one of the practical measures in ensuring the sustainability of beef production (Hocquette and Chatellier, 2011). The concept of residual feed intake (RFI) was introduced by Koch et al. (1963) to account for feed requirements for both maintenance and growth. Aside from the use of RFI to improve feed efficiency, and hence increase profitability in beef production, growth promotants have also been incorporated into beef production systems to improve efficiency, and increase yields (Ebarb *et al.,* 2017). Studies conducted previously demonstrated that growth promotants affect meat tenderness negatively through an increase in the activity of calpastatin (Gerken *et al.,* 1995; Strydom *et al.,* 2009). Meat tenderness is considered to be the most important meat quality trait in relation to eating quality (Miller *et al.*, 2001), and is dependent on numerous physical, chemical and biochemical factors (Christensen *et al.*, 2011). The inconsistent tenderness encountered in beef is one of the main factors that affect consumer satisfaction and has been identified as one of the major challenges faced by the beef industry (Koohmaraie, 1994).

1.1 Beef production

According to the United Nations Food and Agriculture Organization, the global population is expected to reach about 9.5 billion by the year 2050. This increase in world population is expected to be accompanied by an increase in food demand, including the demand for meat. Beef is increasingly becoming the preferred meat around the world, especially as a source of protein (WHO, 2015; Li, 2017; Smith *et al.,* 2018). Sustainability of beef production to meet the increasing demand for meat is mainly determined by factors such as land mass and its associated use, rainfall, accessibility to feed for livestock and the strength of the economy (Smith *et al.,* 2018). Beef producers in Canada are implementing growth promoting technologies and the use of cattle that are more feed efficient, to address industry and environmental sustainability.

Canada is one of the largest global exporters of livestock and red meat, ranking 7th out of the 10 leading exporters of cattle and beef (FAS/USDA, 2020). In 2019, Canada exported 679,600

tonnes of beef and cattle which represented 47% of total live cattle and beef produced (CCA, 2020). Beef consumption in Canada increased by 1.6% to 958,000 tonnes in 2019 (CCA, 2020). The Canadian beef industry provides Canadians with an estimated 228,000 jobs, generating an annual contribution of $18 billion to the gross domestic product (GDP) (CCA, 2020). Considering the projected increase in world population, the increase in the demand for beef is expected to render additional pressure on resources of production which include land, water, labour and feed for food producing animals (Gerber *et al*., 2013). This may lead to competition for land and food resources between man, plants and animals. Furthermore, cattle production has been estimated to contribute to about 65% of total livestock greenhouse gas emissions (Gerber *et al*., 2013). The current impact of cattle production on the environment, coupled with an increase in the demand for beef, calls for efficient and sustainable methods of production.

1.2 Cattle Production Efficiency

Although the present beef cow inventory in the US is about the same as it was in 1965, total beef production has increased from about 13.2 to about 27 billion pounds, representing a doubling of product increase (Elam and Preston, 2004). Additionally, production efficiency has increased from 62.14 to more than 113.40 kilograms of beef per total cow inventory, representing an increase of over 80% (Elam and Preston, 2004). A majority of these gains in production efficiency were achieved by the implementation of production systems that incorporated grain-feeding, reproductive and pharmaceutical-based technologies, as well as the use of crossbreeding and selection programmes that target production traits (Carstens and Kerly, 2009). Growth promoters may have contributed to the doubling of total production of beef in the US in the last 50 years, from 5.99 to about 12.25 billion kilograms (Elam and Preston, 2004). Crossbreeding and selection programmes that concentrate on production traits also have been used although advancement in productivity in beef production was attained without the use of direct selection to boost feed efficiency (Archer *et al.,* 1999; Johnson *et al.,* 2003). Selection for reduced feed intake without compromising the animal's production ability is necessary for increased profitability as feed constitutes up to 70% of total production cost (Cattle Fax, 2017).

A number of ratios for feed conversion have been used to estimate cattle feed efficiency and include the Kleiber ratio (KR), relative growth rate (RGR), partial efficiency of growth, and

feed conversion ratio (FCR). These however do not completely account for the external and internal influences that cause variations in feed conversion (Zhen-qiang *et al*., 2012) and according to Hess et al. (1948), FCR has a low to moderate heritability value. Residual feed intake (RFI) was introduced by Koch et al. (1963) and is essentially the difference between an animal's expected and actual feed consumption adjusted for its production level which include body weight and weight gain, and milk production for a specified test period (Jiu *et al.,* 2020). RFI has been recommended as a true measure of feed efficiency that shows the biological difference in fundamental metabolic processes (Archer *et al.,* 1999). As a feed efficiency trait, RFI evaluates the variation between animals in the intake of feed beyond the amount expected to meet maintenance energy requirement and energy required for production. Hence, when RFI is applied as a selection pressure, it will boost efficiency in feed utilization with limited effect on animal growth or mature size (Carstens and Kerly, 2009). Accordingly, RFI better shows the variation that exists inherently in metabolic processes related to differences between animals in total feed efficiency and not production level. Deviation in RFI in growing beef cattle has been related to variations in heat and methane production, gain composition and digestibility (Carstens and Kerly, 2009), indicating that genetic variation in RFI is due to a number of biological processes (Taussat *et al*., 2020). There is however limited knowledge with respect to how the quality of diet or growth stage affects the genetic ranking of animals for RFI. Further information is needed to understand the biological mechanism that causes differences in RFI among animals, even between those of the same breed.

For several decades, anabolic steroidal growth promotants in the form of hormonal implants have been used in commercial feedlots to improve production performance (Anderson *et al.,* 2010; Ebarb *et al.,* 2017) and efficiency (Eng, 2000). Duckett and Pratt (2014) reported that hormonal implants have the ability to increase average daily gain (ADG), carcass weight and ribeye area by 18, 5, and 4% respectively. The combined improvement from yield and carcass quality may provide producers a profit of about $163 per head (Duckett and Prat, 2014). Hormonal implants may be administered to slaughter cattle to promote growth during the suckling, growing and finishing periods of the production cycle. Steers as well as heifers intended for finishing and harvest may be implanted as many as four to six times, and sometimes more throughout their lifetime (Mader, 1997).

Beta-adrenergic agonists (β-AA) have been approved for use in finishing beef cattle in the last two (2) decades, with ractopamine hydrochloride (RH) being the sole β-AA available for use in North America (Ebarb *et al.*, 2016). Previously referred to as phenethanolamines, β-adrenergic agonists are a class of growth promotants that includes ractopamine hydrochloride (RH), clenbuterol and zilpaterol hydrochloride (ZH). β-AAs have been studied for over thirty (30) years in livestock species as they were shown to increase on lean muscle gain efficiency (Anderson *et al*., 2010). Phenethanolamines have been safely and effectively used in human medicine for over 40 years as bronchodilators in the treatment of asthma, and as uterine relaxants to stop premature labour in pregnant women. They have also been used as cardio-stimulants in the treatment of cardiac irregularities (Ricks *et al*., 1984). RH is approved to be used in cattle in three (3) countries, in swine in twenty-six (26) countries, and in turkey (Elanco Animal Health), while ZH was approved to be used in cattle in eleven (11) countries (Interval/Schering-Plough, Animal Health). RH primarily stimulates β-adrenergic receptors located on muscle cells.

β-adrenergic agonists have been shown to possess no antibiotic activity and therefore do not operate as antibiotic growth promoters, nor are they anabolic steroids (Anderson *et al.,* 2010). As repartitioning agents, β-AAs possess the ability to deflect nutrients away from adipose tissue and direct them towards muscle tissue (Ricks *et al*., 1984). The general effects of β-adrenergic agonists include enhanced efficiency of feed utilization, an increase in lean deposition, an increase in rate of weight gain, and an increase in dressing percentage, and all of these outcomes have been demonstrated in beef cattle, swine, lambs, broilers and turkey (Anderson *et al.,* 1991). Additionally, a meta-analysis conducted by Lean et al. (2014) stated that zilpaterol hydrochloride (ZH), another β-AA, increased ADG, carcass weight and ribeye area by 0.15 kg, 15 kg and 8 cm^2 respectively. RH has been shown to increase *longissimus* muscle area (LMA) (Lean *et al.,* 2014) with limited effect on adipose tissue (Mersmann, 1998). Diet, dosage, duration of supplementation, age, weight and genetics have been reported to influence response to β-AAs and are therefore crucial to the success of incorporating it in the production of livestock (Anderson *et al.,* 2010). Although RH is primarily a β_1-adrenergic receptor agonist, it has binding affinities for both β_1- and β_2- adrenergic receptors (Colbert *et al.,* 1991; Moody *et al.,* 2000). A number of studies have shown that the use of RH increases the rate of animal growth, as well as the production of red meat (Elam *et al.,* 2009; Boler *et al*., 2012; Arp *et al.,* 2014). According to Bryant et al. (2006), growth

in the finishing stage can be improved in continuous phases by the use of anabolic hormonal implants and β-adrenergic agonists which together produce favourable additive effects on cattle performance. This unfortunately results in reduced marbling and tenderness, hence reduced carcass quality grade (Dikeman, 2007). Although a number of studies have investigated interactions between ractopamine hydrochloride and anabolic hormonal implants on production performance and meat quality, the results are contradictory (Moloney *et al*., 1990; Bass *et al*., 2006).

β-adrenergic agonists are orally active and dispensed as ingredients in feed. Abney *et al.,* (2007) and Boler *et al.,* (2012) reported that supplementation with RH can lead to an increase in average daily gain (ADG) and gain to feed ratio (G:F) during the last 28 to 42 days in finishing cattle without a change in dry matter intake (DMI). There is however insufficient information on how RH influences the feeding behaviour of finishing cattle (Trotta *et al.,* 2019). The study by Boler et al. (2012) showed that steers supplemented with RH at a rate of 300 mg/head/day for a duration of 28 days exhibited more feeding events that were brief and smaller in quantity. On the other hand, when steers were supplemented with RH at a rate of 200 mg/head/day for 30 days, the time required to consume 50% and 70% of the daily feed allowance was prolonged (Abney *et al.,* 2007). There are no studies on how protracted RH supplementation, such as for 42 days, influences feeding behaviour in steers (Trotta *et al.,* 2019). RH supplementation has been shown to activate feed intake in ruminants, likely through an increase in the production of γ-aminobutyric acid in the brain (Baile and McLaughlin, 1987). The dose and the duration through which the dose is sustained determines the optimum response to the supplementation of the β-adrenergic agonist (Johnson *et al.,* 2014). Typical supplementation of RH has however been reported to have no effect on DMI (kg/d) of steers (Abney *et al*., 2007; Lean *et al*, 2014). In the study by Trotta et al. (2019), results showed that supplementing finishing steers with RH at a daily rate of 267 mg/head did not affect DMI (kg/d). However, a change in feeding behaviour, where an increase in DMI per minute accompanied by a subsequent reduction of 13.7% per day in eating time was reported. For carcass characteristics, Trotta et al. (2019) reported a reduction in marbling, which corroborates reports by Gruber et al. (2007) and Winterholler et al. (2007). Despite the fact that the biological importance of the effect of RH supplementation on the rate of DMI remains inconclusive, there are concerns associated with how feed is managed to prevent disturbances in the digestive system of steers supplemented with RH, such as rumen acidosis. Further research is needed to investigate how RH

supplementation affects feeding behaviour, and particularly its ability to influence fermentation in the rumen, and the function of the gastrointestinal tract as a whole.

1.3 Growth Promotion and Beef Quality

It is worth noting that some researchers have suggested that aggressive and/or multiple implantations may negatively impact some meat quality characteristics such as tenderness (Morgan, 1997; Roeber *et al*., 2000). The National Beef Quality Audit in the U.S raised concerns about three decades ago regarding the potential for hormonal implants to reduce marbling scores and ultimately meat tenderness (Smith *et al.*, 1992). Roeber et al. (2000) and Platter et al. (2003) found that steaks obtained from implanted steers had significantly higher Warner-Bratzler Shear Force (WBSF) value than steaks obtained from non-implanted steers. They further reported that the closer to their time of slaughter the steers were implanted, the more likely the implants were to increase shear values. More recently Ebarb et al. (2017) found increased beef toughness in implanted steers. A survey of leading beef packers in the United States during the 2005 National Beef Quality Audit stated that reduction in quality grades and tenderness as a result of implant utilization was rated first among the primary ten meat quality problems faced by the beef industry in the United States (Smith *et al*., 2006). A similar phenomenon was reported for β-AA, as Leheska et al. (2009) stated that ZH increased WBSF in heifers by 24%. Although numerous studies have been performed on the effect of growth promotants on the eating quality of meat, particularly beef, the exact biological mechanism involved in their negative impact on tenderness largely remains unknown (Woerner *et al*., 2011; Boles *et al.,* 2009; Ebarb *et al*., 2017). Roy et al. (2015) and Ebarb et al. (2017) hypothesized that the use of growth promotants impacted meat tenderness negatively by increasing the mature trivalent crosslinks between collagen molecules, and increasing the cross-sectional area of muscle fibres respectively.

To understand the implications of growth promoting technologies on beef quality, the ways in which beef quality are estimated and valued need to be considered. Systems of grading beef have been developed as a method of classification of carcasses according to anticipated meat quality, with carcasses having similar characteristics grouped into grades. Beef grades in Canada serve as a trade language, enabling production and marketing decisions, as well as an inference of meat-eating quality (Aalhus *et al.*, 2014). This provides an assurance that allows consumers to

purchase beef products that are predictable as well as consistent (Aalhus *et al.*, 2014). More than 90% of the total number of cattle slaughtered in Canada are graded by the Canadian Beef Grading Agency, with the remaining 10% not graded and referred to as "no roll" (ungraded) beef as they are mainly derived from slaughtered animals in mature populations (CanFax, 2012). Carcasses from young animals in Canada are characterized by less than 50% ossification in the spinous process of the vertebrae. This qualifies them for the A or B beef grades (Canada Gazette, 2007; Canadian Beef Grading Agency, 2014). Carcasses deemed youthful have backfat thickness that is more than 2 mm but less than 15 mm, have slight amounts of marbling, muscling that ranges from good to excellent, meat colour which is bright red, and fat which is white to amber in colour are eligible to be graded as A (Aalhus *et al.*, 2014). Carcasses considered youthful that meet government criteria for texture, muscle and composition, and colour are categorized into four quality A grades in Canada. These beef quality grades are assigned based on the observable amount of intramuscular fat (marbling) in the *longissimus thoracis* (LT) (rib-eye) muscle at the interface of the 12th and 13th rib. According to this system of grading, Canada A is characterized by a trace amount of intramuscular fat, with slight marbling classified as Canada AA, and small amount of marbling classified as Canada AAA. The grade Canada Prime is assigned when there is abundant marbling or greater (Aalhus *et al.*, 2014). According to Agriculture and Agri-Food Canada (2014), Canada A, AA, AAA and Prime are equivalent to the USDA Standard, Select, Choice and Prime, respectively. This allows for convenience in trade as about 75% of the beef export market of Canada lies in the USA.

1.3.1 Meat colour, pH and Temperature

Meat colour is a beef quality attribute that is considered important by consumers (Juszczuk-Kubiak *et al.,* 2007) and is an important determinant in the Canadian Beef Grading system because only beef with a bright red colour is eligible for the quality grades. The perception of high nutritive value, low fat content and freshness of meat by consumers also play an important role in the quality of meat (Resurreccion, 2004). Meat colour depends on the concentration and chemical state of myoglobin located in the muscle tissue. At the point of sale, consumers use fresh meat colour as a measure of wholesomeness while cooked meat colour is used to determine its doneness at the point of consumption (Suman and Joseph, 2013). Thus, meat colour is the most important meat quality attribute at the point of sale as it influences consumers' purchasing power (Mancini and Hunt,

2005). Consumers generally are unable to assess the aroma or texture of meat while they are packaged. A bright cherry-red colour is therefore regularly used as a measure of wholesomeness in fresh meat. When the surface of whole-muscle cuts is discoloured, they are ground into products of low value such as ground beef or are discarded before they become microbially unsafe. All these steps ultimately lead to losses in sales and wastage of otherwise valuable food (Faustman and Cassens, 1990). Price discounts of meat influenced by discoloration has been estimated to be over a billion dollars each year for the meat industry in the U.S (Smith *et al.,* 2000). Although meat wastage due to discoloration negatively affects agricultural sustainability and the environment, its full impact is yet to be determined (Suman and Joseph, 2013).

The sarcoplasmic heme protein that is the principal factor responsible for meat colour is myoglobin (Suman and Joseph, 2013). Myoglobin can exist in different forms in fresh meat. Deoxymyoglobin presents in freshly cut meat, imbuing it with a deep purplish-red colour. Upon exposure to oxygen, deoxymyoglobin is oxygenated to oxymyoglobin which gives meat a bright pinkish red colour. Deoxymyoglobin and oxymyoglobin can both be oxidized to metmyoglobin, which is given meat a brown colour. Factors such as low pH, high concentration of salt and UV light are advantageous for the formation of metmyoglobin (Purslow, 2005).

The colour of beef can indicate the quality of the meat as affected by the decline of muscle pH and temperature in the early *post-mortem* period. Normal beef with a cherry-red colour has a pH range from 5.4 to 5.59 (Page *et al.,* 2001). Muscles that have a pH value greater than 5.7 tend to be dark (Orcutt *et al.,* 1984). Low pH coupled with high temperature however improves muscle protein denaturation. This further leads to the increase of open muscle structures and results in an increase in the penetration of oxygen and oxymyoglobin (OMb) formation (Farouk and Lovatt, 2000). The consumption of oxygen by the mitochondrion is diminished at low muscle pH (Tang *et al.,* 2005), due to the inactivation of electron transport system enzymes in the inner mitochondrial membrane (Bendall and Taylor, 1972). The low consumption of oxygen at low pH increases OMb which enhances muscle colour (Young *et al.*, 1999; Sammel *et al.,* 2002). Bruce and Ball (1990) found that *post-mortem* ageing of muscles at 31 ℃ for 4 hours, 12 ℃ for 4 hours, 7 ℃ for 12 hours and 2 ℃ for the remaining time decreased intramuscular pH, sarcomere length and protein solubilities, while the fragmentation index shear force and colour reflectance were increased. The pH and temperature of muscle continuously interact during the development of

rigor to affect sarcomere physical shortening (Tornberg, 1996), as well as the activity of proteolytic enzymes (Koohmaraie *et al.*, 1986; Dransfield, 1992). In a study by Hwang and Thompson (2001), rapid glycolysis or slow chilling of carcasses resulted in a decrease in the level of activity of μ-calpains at 4 hours *post-mortem*. This will potentially influence meat tenderness negatively.

1.3.2 Meat tenderness

Meat tenderness is determined by myofibrillar structures and the connective tissues that surround them, the latter being mainly composed of collagen (Nishimura, 2010). The intrinsic meat quality characteristics, including tenderness, are dependent on the characteristics of the muscle in the live animal, and also on factors *post-mortem,* such as ageing period and cooking methods (Nishimura, 2010). Muscle fibres consist of myofibrils composed of thin actin and thick myosin filaments that are responsible for the actomyosin toughness of meat (Nishimura, 2010). During the *post-mortem* period when meat is aged, the structural integrity of the muscle fibres is compromised by proteolysis. This contributes to the tenderness observed with aged meat (Dransfield *et al.,* 1984; Takashi *et al.,* 1996). Meat quality characteristics are also influenced by genetic, nutritional and management factors (Cassar-Malek *et al.,* 2008). According to Wheeler et al. (1994), marbling has been shown to influence tenderness in a small but positive manner. May et al. (1992) demonstrated that marbling score is moderately correlated with tenderness as perceived by a sensory panel ($r = 0.51$) and as measured using shear force ($r = -0.61$) in Angus × Hereford crossbred steers, a breed cross that is associated with a relatively high marbling ability. Another breed known to deposit large amounts of intramuscular fat is the Japanese Black (Zembayashi, 1994), a breed in which was found a high inverse correlation coefficient between crude fat content and shear force value of raw *longissimus* muscle after 20 months of age (Nishimura *et al*., 1999). This presents a trend where a higher degree of marbling is associated with meat tenderness (Nishimura, 2010). Delayed cooling of muscles insulated by back fat may lead to an increase in proteolysis of the muscle fibres, and increased meat tenderness (Lochner et *al.,* 1980), but the increase in shear force in muscles aged at high temperature in the study by Bruce and Ball (1990) is explained by the potential for high-temperature ageing (30 to 40 °C) (Busch *et al.,* 1967) to cause heat shortening (Lee and Ashmore, 1985). This may further affect

tenderness negatively, through shortening of sarcomeres and decreased protein solubility (Koh *et al.,* 1987).

Growth promotants are known to redirect nutrients towards the deposition of lean tissue in carcasses, away from adipose tissue (Bradley and Chung, 2007). According to Bradley and Chung (2007), growth promotants that are commonly used in beef production include steroidal implants and β-AAs, and have collectively been shown as a contributing factor of reduced scores of marbling in beef cattle. In a study by Johnson et al. (1996), steers treated with trenbolone acetate/estradiol (TBA/E_2) implants had an increase in carcass weight from 18.14 to 27.21 kg, and rib-eye area from 0.5 to 1.0 in^2, without any effect on fat thickness, when compared with untreated steers, fed for the same number of days. Regardless of the concerns about the negative effect of steroid implants on marbling and quality grades of beef carcasses, anabolic implants are routinely used in the finishing phase in the production of beef, to enhance body gain and feed efficiency (Duckett *et al.,* 1996). The use of steroid implants has been responsible for the reduction in the number of carcasses that grade as Choice (Belk, 1992). A review of 37 trials involving implanted steers on finishing diets showed that marbling was reduced by 24%. According to Duckett et al. (1996), the number of carcasses that grade choice reduced by 14.5% when steers were implanted. The authors also reported a difference ranging from -72 to + 31% in marbling, and from -50 to + 9% in carcass grading Choice. An increase in protein accretion, a decrease in proportional fat content, as well as an increase in moisture content in beef carcasses have been associated with the use of the β-AA ractopamine hydrochloride (Parrot *et al*., 1990; Schroeder *et al.,* 2003). According to Winterholler et al. (2007) and Gruber et al. (2007), marbling scores were reduced by 10% in cattle fed RH at a rate of 200 mg compared with untreated cattle. Trotta et al. (2019) also reported reduced marbling scores in steers treated with the RH. In a study by Bittner et al. (2017) however, carcasses from RH treated and untreated steers did not differ in marbling score. Due to conflicting reports on marbling scores as a result of RH treatment, further studies are warranted to ascertain the true effect of β-AA on marbling.

1.4 Intramuscular connective tissue

Physiologically, intramuscular connective tissue (IMCT) functions as a scaffold to support muscle tissue, and conducts forces that are generated by contractile proteins (actin and myosin) to

the skeleton through tendons. The connective tissue is therefore very resilient in order to play this role (Stanton and Light, 1987). IMCT is formed concurrently with muscle fibres during embryonic development. Postnatally, it is reconstructed to accommodate the addition of myofibrils to muscle fibres during muscle hypertrophy and the deposition of intramuscular fat (IMF) (Nishimura, 2010). These structural changes influence the mechanical strength of the IMCT and further contribute to variation in the texture of meat (Nishimura, 2010). Three layers of intramuscular connective tissue, namely endomysium, perimysium and epimysium, maintain the structural integrity of skeletal muscle fibres. The endomysium surrounds individual muscle fibres, the perimysium surrounds bundles of muscle fibres while the epimysium surrounds the whole muscle (Mayne, 1982; Nishimura, 2010). The components of connective tissues include cells of the extracellular matrix (ECM), which is mainly composed of collagen and makes up 95% or more of the fibrous constituent of the connective tissue, while elastin, proteoglycans and minor glycoproteins make up the residual 5% (Stanton and Light, 1987; Nishimura, 2010). As one of the most abundant proteins found in the bodies of mammals, collagen is the fundamental protein of the skeletal muscle, and its associated connective tissues (Stanton and Light, 1987). Collagen is present mainly in the skin, bones, cartilages, tendons, and teeth (Taffin and Pluvinet (2006), forming the major constituent of several essential tissues, and is crucial in maintaining the structure and function of the body (Sandhu *et al.,* 2012). Currently, 28 different types of collagen have been identified (Shoulders and Raines, 2009).

The IMCT is mainly composed of collagen (Light, 1987), with the perimysium accounting for about 90% of total IMCT collagen (McCormick, 1994). Collagen varies in its amount, composition and structure between different breeds, sex and animal age (Boccard *et al.*, 1979). Much research attention has been given to the myofibrillar contribution to meat tenderness as opposed to contribution by the IMCT. This is because during *post-mortem* ageing of meat, the IMCT had previously been considered to be immutable compared to myofibrils (Purslow, 2005), and was thus referred to as the background toughness of meat (Nishimura, 2010). In cooked beef, the strands of the perimysium have higher tensile strength than junctions between the perimysium and the endomysium (Lewis and Purslow, 1990). These findings suggest that the constitution of the perimysium remains a major factor that influences meat tenderness (Swatland *et al.,* 1995). Poor correlations however have been shown between Warner-Bratzler Shear Force (WBSF) of cooked meat and the content of the perimysium (Brooks and Savell, 2004). Furthermore, meat

toughness has been shown to increase or decrease depending on cooking temperature, with the perimysial connective tissue strength shown to increase in meat cooked to a temperature of 50 °C. Above a cooking temperature of 50 °C, the tensile strength of the perimysium decreases (Lewis and Purslow, 1989). These results make cooking temperature an important contributing factor to cooked meat toughness or tenderness.

Collagen molecules are made of three polypeptide chains known as α-chains. They contain a minimum of one domain which consists of Gly-X-Y sequences in a repeating manner in all of the constituting chains. Some collagen molecules contain identical α-chains while other molecules contain two or at times three different α-chains. The three α-chains are individually coiled into a left-handed triple helix. Glycine, which occurs in every third position and is the smallest amino acid, is crucial for the arrangement of the coiled-coil structure. This is because as the smallest amino acid, glycine permits the tight turn of each α-chain in order to provoke the structure of the triple helix (Myllyharju and Kivirikko, 2004; Shoulders and Raines, 2009). Aside from glycine, any other amino acid can occupy the X and Y positions. However, the X position is usually occupied by proline, while 4-hydroxyproline is often found in the Y position (Myllyharju and Kivirikko, 2004). The amino acid 4-hydroxyproline is important in collagen synthesis as it functions to stabilize the triple helix (Myllyharju and Kivirikko, 2004). This is achieved through an inductive effect of the hydroxyl group of 4-hydroxyproline to stabilize the *trans* X-Hyp conformation and strengthen the hydrogen bond between them (Holmgren, 1998; Jenkins and Raines, 2002).

Collagen, as a major constituent of connective tissues garners its strength from the crosslinks it forms between its molecules with the crosslinks formed between collagen molecules determining its heat solubility (Tanzer, 1973). Lysine- or hydroxylysine-derived aldehydes found in the non-helical regions of the collagen molecule initially react with their unmodified counterparts in adjoining molecules. This occurs between helical and non-helical locations and form Schiff base divalent crosslinks (Eyre *et al.*, 1984). The divalent crosslinks are acid-labile and decrease in concentration as an animal ages until nearly absent at maturity (Shimokomaki *et al.*, 1972). It is thought that the divalent crosslinks, which are reducible, undergo further reaction that converts them to non-reducible trivalent crosslinks (Kuypers *et al.,* 1994). Pyridinoline (Pyr) and Ehrlich's chromogen (EC) are two non-reducible trivalent crosslinks have been isolated and

described (Fujimoto 1977; Scott *et al*., 1981). Pyr is a 3-hydroxypyridinium compound (Eyre *et al*., 1984) that is found as hydroxylysyl-pyridinoline and lysyl-pyridinoline in IMCT. Hydroxylysyl-pyridinoline constitutes 90 to 95% of total Pyr while lysyl-pyridinoline constitutes the remainder of 5 to 10% (Bosselmann *et al.*, 1995; Steinhart *et al.*, 1994). Ehrlich's chromogen (EC), which is pyrrolic (Kuypers *et al.,* 1992), is thought to be an N-substituted pyrrole (Scott *et al.*, 1981).

Studies on the location of Pyr in cartilage, bone and aorta reveal two sites for the formation of Pyr (Robins and Duncan, 1983; Robins and Duncan, 1987; Henkel *et al*., 1987). Each of the two sites link three collagen chains, two of which include the aldehyde-forming lysine residues located in the N- or C-terminal non-helical regions with hydroxylysine at the C- or N-terminal helical regions, respectively, of a third adjacent quarter-staggered chain (Kuypers *et al.,* 1992). The presence of EC was well exhibited in a three-chain cross linked peptide from human type III collagen (Scott *et al.*, 1983). The mechanisms proposed for the formation of EC and Pyr are well described by Eyre and Wu (2005). Inconsistent with reports from previous studies on EC crosslinks in type III collagen isolated from leiomyoma tissues in humans however, all EC crosslinked peptides found in the study by Kuypers et al. (1994) developed from type I collagen. Formation of EC evidently does not occur with type III collagen of the perimysium (Kuypers *et al.,* 1994). This can be explained by the mechanism of formation of EC and Pyr proposed by Kuypers et al. (1992). According to Kuypers et al. (1992), the formation of Pyr and EC results from a reaction involving a bifunctional ketoamine crosslink and a free hydroxylysine-derived or lysine-derived aldehyde. Thus, if aldehydes that are free in type III collagen in the perimysium were all derived from hydroxylysine, the only trivalent crosslink that would form in collagen type III would be Pyr. Likewise, for the formation of EC to transpire solely with type I collagen, there would be a requirement for all free aldehydes to be derived from lysine. This proposed mechanism is supported by the observation that collagen types I and III co-polymers are crosslinked only by Pyr

According to Steinhart et al. (1994), the amount of Pyr in a muscle fluctuates with total collagen concentration of the muscle. This has been further implicated in reduced heat-solubility of collagen, and hence increased meat toughness (Reiser *et al*., 1992; Xiong *et al.*, 2007) as an animal matures. In the different muscles of the animal, changes in intramuscular collagen occur at varying rates (Kolczak *et al.,* 1992). According to Light et al. (1985), tender beef has fewer

crosslinks between its collagen molecules found in the endomysium and the perimysium. A study by Lepetit (2007) showed that the total amount of crosslinks found in a given volume of cooked meat is almost proportional to the elastic modulus of collagen fractions in the connective tissue. Thus, the effect of crosslinks on the toughness of meat is additive. However, the correlation between collagen crosslinks and meat tenderness remains inconclusive (Nishimura, 2010). According to Dubost et al. (2013), collagen transforms to gelatin when cooked, which improves meat tenderness. The presence of mature trivalent collagen crosslinks however hinders collagen gelatinization which leads to the production of tough meat in mature animals.

Muscle function is another factor that influences collagen crosslink density. Muscles involved in locomotion contain more covalent collagen crosslinks compared to postural muscles (Zimmerman *et al.*, 1993). In muscles found in different locations in the same animal, variations in the turnover of collagen may differ which ultimately influences collagen cross-linking and therefore meat tenderness in the different muscles (Archile-Contreras *et al.,* 2010). In positional muscles, such as the *longissimus thoracis et lumborum*, the concentration of collagen as well as the number and density of its associated heat-stable crosslinks are minimal. This mostly results in the positional muscles being more tender as opposed to locomotive muscles, which have higher collagen concentration and more heat-stable collagen crosslinks, and are mostly tough (Dubost *et al.,* 2013).

Total collagen can also contribute to the toughness and price of beef, with muscles with low amounts of collagen commanding the highest prices (Bruce and Roy, 2019). In the study by Boccard et al. (1979), collagen content was determined in muscles from bulls of two breeds at six different ages, at birth, 8, 12, 16, 20 and 24 months of age. In the study, collagen content was higher at birth in muscles from both breeds compared to all other ages. This corroborated reports by Hill (1968) and Vognarova et al. (1968), and can be explained by earlier development of proteins of the connective tissue than muscle contractile proteins (Boccard *et al.*, 1979). Between 8 and 12 months of age, however, an increase was observed in total collagen content in two muscles with high collagen content, namely the *pectoralis profundus* (PP) and *triceps brachii* (TB). Constant collagen levels were attained and maintained form 16 months onwards. The increase in the level of collagen between 8 and 12 months of age was associated with an evident sexual development in young bulls, and is an indication of hormonal activity of young male

animals (Hafez, 1972). Higher amounts of mature collagen crosslinks are found in muscles from older animals which are less tender while lesser amounts of collagen crosslinks are found in muscles from younger animals, which are also more tender (Jurie *et al.,* 2005; Calkins *et al.,* 2007).

1.5 Enzymes and muscle fibre proteolysis

How growth promotants increase beef toughness is not fully understood, but most hypotheses focus on the myofibrillar protein accretion in the live animal and the potential for proteolytic degradation *post-mortem*. The level of proteolysis of major target proteins within muscle fibres seems to be the key determinant of ultimate meat tenderness (Taylor *et al*., 1995a; Koohmaraie and Geesink, 2006). According to Hopkins and Taylor (2002), ultimate meat tenderness is dependent on the level of modification of the structure of the muscle, and its related proteins. Titin, desmin and vinculin, which represent specific myofibrillar, cytoskeleton and costamere proteins, respectively, undergo cleavage. There is also considerable cleavage of actin and myosin which are the major myofibrillar proteins (Goll *et al*., 1992; Taylor *et al.,* 1995a; Koohmaraie and Geesink, 2006).

Four proteinase systems have been examined for their potential to degrade myofibrillar proteins *post-mortem*, specifically the cathepsins, the proteasome, the calpains and the caspases. The cathepsins belong to a group of exo- and endo-peptidases. They are classified into cysteine (cathepsins B, H, L and X), aspartic (cathepsins D and E) and serine (cathepsin G) families of peptidases (Sentandreu *et al*., 2002). The contribution of cathepsins to meat tenderization has been rejected by a number of researchers as they do not remarkably degrade actin and myosin during the ageing period (Koohmaraie *et al*., 1991). Additionally, cathepsins are found in lysosomes and need to be released to access myofibrillar proteins and contribute to meat tenderization (Hopkins and Taylor, 2002). Low levels of pH and high carcass temperature however may promote the disruption of the membrane of lysosomes (O'Halloran *et al.,* 1997). Furthermore, the failure of pumps in membranes as a result of rigor as adenosine triphosphate (ATP) is depleted may present a means of resolving this challenge (Hopkins and Thompson, 2002). Overall, limited association has been established between the activities of cathepsins and variation in tenderness (Whipple *et al.,* 1990). Regardless, a positive correlation has been found between the activities of B and L cathepsins and beef tenderness at 8 hours *post-mortem* (O'Halloran *et al.,* 1997).

Proteasomes degrade proteins found in the cytosol and nucleus, and are multi-catalytic protease complexes that are implicated in controlling numerous basic cellular pathways (Coux *et al.,* 1996). According to Robert et al. (1999), proteasomes are expressed widely in the body and are extensively present in the skeletal muscle. The proteasome (26S) constitutes a 19S regulatory subunit and a 20S structure which is multi-catalytic. Accordingly referred to as the multi-catalytic proteinase complex, the 20S proteasome is the catalytic core of the proteasome complex (Dahlmann *et al*., 2001). The proteolytic activity of the proteasome depends on a process regulated by ubiquitin. A minimum of four ubiquitin proteins are required to attach to the lysine residue of target substrates. This makes the substrate recognizable by the proteasome which removes the chain of ubiquitin and finally degrades the substrate (Taillandier *et al.,* 2004). ATP is required for this process and when it is exhausted the 26S proteasome dissociates into the 19S subunit and 20S proteasome. The 20S proteasome does not have a requirement for ATP or ubiquitin (Peters *et al*., 1994). The activity of proteasomes is however modulated by the binding of different proteasome activators or regulatory particles which include 19S, and proteasome activators 28 (PA28) and 200 (PA200) (Mao *et al.,* 2008; Cascio, 2014). The regulatory particles have the ability to bind symmetrically and asymmetrically to the α-rings of the 20S core, leading to the formation of single or double capped proteasomes (Fabre *et al.*, 2014). The main proteasome activator is the 19S subunit, which forms the 19S-20S (26S) proteasome complex (Fabre *et al.*, 2014). This cap is important in the ubiquitin-proteasome system, which is a pathway responsible for the degradation of misfolded and regulatory proteins including short-lived regulators of cell cycle and activators of transcription (Glickman and Ciechanover, 2002). Following binding of a substrate to a proteasome, the 19S regulatory particle deubiquitinates, trans-locates and unfolds the substrate protein in a manner that is dependent on ATP. This allows the substrate protein to be degraded by the 20S core (Navon and Goldberg, 2001; Collins and Goldberg, 2017). Early studies on the proteasome reported that the enzyme was not implicated in the proteolysis of the myofibril (Koohmaraie, 1992). A number of ensuing studies have however shown that the proteasome is capable of contributing to meat tenderization (Taylor *et al.,* 1995b). A study by Robert et al. (1999) in bovine myofibrils reported that proteasomes in bovines have the ability to hydrolyze myofibrillar proteins such as nebulin, myosin, actin and tropomyosin. Additionally, in a study by Lamare et al. (2002), the activity of the proteasome was found to be maintained, with a significant amount of activity still observable at 7 days *post-mortem* and at a pH below 6.

According to Koohmaraie and Geesink (2006), calpains remain the most widely studied family of proteases in meat science. It has therefore been largely accepted that the activity of the proteolytic calpain contributes to meat tenderness. Calpains are part of a family of intracellular cysteine proteases (Shimada *et al.,* 2008). The calpain system in the skeletal muscle is made up of μ-calpain, m-calpain and calpain 3/p94. The μ- and m-calpains are expressed ubiquitously and activated by Ca^{2+}, in micro and millimolar concentrations, respectively (Goll *et al.,* 2003). Calpastatin is an endogenous inhibitor of calpain (Wendt *et al*., 2004). Significant evidence connects tenderization in beef, pork and lamb to calpains. Additionally, the ratio of calpastatin to calpain have been shown to be inversely correlated to various rates of tenderization between species where pork had the highest tenderization rate, followed by lamb, and lastly beef (Koohmaraie *et al.,* 1991). According to Bohorov et al. (1987), β-AAs were observed to substantially minimize degradation of muscle protein. Animals supplemented with β-AAs had an increase in activity of calpastatin with a corresponding increase in mRNA expression in the muscle. This suggests that the activity of calpain is implicated in the turnover of muscle protein (Higgins *et al.*, 1988; Bardsley *et al.,* 1992; Killefer and Koohmaraie, 1994). Calpain 3 (p94) is mostly expressed solely in the skeletal muscle (Sorimachi *et al.,* 1989), and binds to titin, which is a major myofibrillar protein at the N_2 line (Sorimachi *et al.,* 1995). The N_2 line is the site of proteolysis which has been connected with meat tenderization (Taylor *et al.,* 1995a). A study by Parr et al. (1999) in *post-mortem* proteolysis and meat tenderization in pigs observed no relationship between the expression of calpain 3 and shear force value at 8 days *post-mortem* in the *longissimus thoracis et lumborum* muscle. In sheep however, strong correlations have been reported between variations in the levels of calpain 3 mRNA and associated proteins, and variations in tenderness (Ilian *et al*, 2001). Muscle fibres incubated with calpains have been observed to follow similar patterns of degradation as observed in the muscle *post-mortem*, where calpains degrade major myofibrillar proteins of which nebulin, titin, troponin-T and desmin are included (Huff-Lonergan *et al.,* 1996). The μ- and m- calpains cleave the same myofibrillar proteins. The μ-calpain is however activated during early *post-mortem* period, specifically within 3 days of slaughter, which is the period when most proteolysis of major proteins of the myofibril occur *post-mortem* (Taylor *et al*., 1995a). The activity of m-calpain is maintained for a longer period than that of the μ-calpain, which is less stable (Sensky *et al.,* 1996). This suggests that the m isoform of calpain is not activated during early *post-mortem*, and that the concentration of Ca^{2+}

in the *post-mortem* muscle is insufficient to activate it (Boehm *et al.,* 1998). Although the early *post-mortem* activity of the calpain system has been found to substantially influence final meat tenderness, it has been found not to be the only proteolytic system to determine meat quality (Kemp *et al.,* 2010).

Caspases belong to a family of cysteine aspartate-specific proteases. About 14 members of the caspase family have been identified, and can further be grouped based on the roles they perform, which could either be apoptotic or inflammatory (Earnshaw *et al.,* 1999). According to a model proposed by Ouali et al. (2006), there may still be activity by the protease family of caspases *post-mortem* which leads to meat tenderization. Apoptotic caspases are also additionally put into sub-groups of initiator caspases (caspases 8, 9, 10 and 12) or effector caspases (caspases 3, 6 and 7). These sub-groupings are dependent on the location of the caspases in the pathway of cell death. The activation of caspases occurs through three main pathways. The extrinsic pathway, also referred to as the cell death pathway is stimulated by receptors found on cell surfaces. As an example, the initiator caspases 8 and 10 are activated through the cell death pathway (Boatright and Salvesen, 2003). Caspase 9 is found in the intrinsic pathway and is activated as a result of response to stresses from the environment such as hypoxia and ischemia (Earnshaw *et al.,* 1999). The pathway mediated by the endoplasmic reticulum (ER) is activated through direct stress on the ER. These stresses include Ca^{2+} homoeostasis disruption, which leads to the activation of caspase 12, which is an initiator caspase. Initiator caspases activate effector caspases which then aim at and cleave distinct substrates, leading to the break-up of the cell (Fuentes-Prior and Salvesen, 2004). About 280 targets of caspases have been identified which include myofibrillar proteins such as α-actinin, filamin, and troponin T, and cytoskeletal proteins such as β-actin, fodrin, and β-spectrin (Fisher *et al.,* 2003).

1.6 Mechanism of action of growth promotants

1.6.1 Hormonal growth promotants (steroids)

To maintain the population of satellite cells needed to support muscle hypertrophy in mature animals, cells in a state of quiescence must be activated to proceed through the cell cycle and contribute nuclei to the growing muscle fibre. Upon activation of quiescent satellite cells, growth

factors with the ability to stimulate proliferation and subsequent differentiation of the satellite cells are needed. Insulin-like growth factor (IGF)-1 and fibroblast growth factor-2 (FGF2) are two known progression factors, as they possess the ability to aid in progressing cells through the cell cycle. These growth factors are known to stimulate the proliferation of satellite cells (Allen and Rankin, 1990; Johnson and Allen, 1990). Studies have shown that treatment with a TBA/E_2 combination implant results in an increase in circulatory levels of IGF-1 and expression levels of IGF-1 mRNA in the *longissimus* muscle of implanted steers in comparison to non-implanted steers 30-40 days after implant use (Johnson *et al.,* 1996; Johnson *et al*., 1998; Dunn *et al.,* 2003).

According to Roeder et al. (1986), anabolic steroids indirectly stimulate protein synthesis in muscle cells of rats. Other studies have also demonstrated that estrogens stimulate the secretion of growth hormones, and increase the number of growth hormone receptors in the liver (Trenkle *et al.,* 1983; Brier *et al.,* 1988; Dayton *et al.,* 1999). Increased production of IGF-I as well as a higher number of activated muscle satellite cells undergoing proliferation are associated with muscle growth induced by steroids (Johnson *et al.,* 1996; Johnson *et al*., 1998). Significantly increased concentrations of circulating levels of IGF-I and steady-state IGF-I mRNA were found in implanted (TBA: E_2) steers when compared to non-implanted steers (Johnson *et al*., 1998). As such, the muscle tissue may produce higher concentrations of IGF-I in implanted (TBA: E_2) steers (Johnson *et al.,* 1998). Additionally, the abundance of steady-state IGF-I mRNA was higher in implanted steers than in non-implanted steers in the same muscles. Furthermore, a larger number of actively proliferating satellite cells were isolated from the *semimembranosus* (SM) muscle of implanted steers than from non-implanted steers (Johnson *et al.,* 1998). Other growth factors that may be involved in muscle growth induced by steroids are the hepatocyte and fibroblast growth factor (Dayton *et al.,* 1999). Increased IGF-1 has been associated with reduced protein degradation (Johnson *et al.,* 1996). Hormonal growth promotants improve the feed conversion ratio (FCR) and growth rates of cattle by reducing the rate of protein turnover (Dikeman 2007; Cafe *et al*. 2010), with protein accretion exceeding protein degradation (Boehm *et al.,* 1998).

Past literature has shown that growth promoting technologies have undesirable effects on meat quality, especially tenderness, due to elevated calpastatin activity (Gerken *et al.,* 1995; Strydom *et al.,* 2009). According to Ebarb et al. (2016), the decrease in tenderness attributed to the use of growth promoters (GP) was as a result of higher cross-sectional area (CSA) of muscle

fibres. The authors further stated that collagen solubility was unaffected when GP were implemented. A study by Roy et al. (2015) showed that when steers were implanted, the amount of hydroxylysyl pyridinoline (HP) crosslinks within the *gluteus medius* (GM) and *semitendinosus* (ST) muscles increased, while supplementation with ractopamine hydrochloride (RH) did not affect the content of HP. The hormone testosterone has been implicated in an increase in calpastatin, a protein inhibitor of the proteolytic enzyme calpain (Morgan *et al*., 1993). Although steroid implants have been shown to decrease beef tenderness, it is not known if animals with larger frames that grow at a rapid rate would have different tenderness measures from animals with smaller frames within the same breed (Nichols *et al*., 2002). Animals that are heavily muscled and late maturing may have a larger rate of protein accumulation, as well, that is coupled with a reduction in degradation due to increased activity of calpastatin. As growth implants increase the natural rate of muscle growth in animals, they may further aggravate issues of toughness associated with these animals (Boles *et al.,* 2004).

1.6.2 βeta-Adrenergic Agonists

How β-AA would decrease beef tenderness is also not well characterized. According to Avendaño-Reyes et al. (2006) and Scramlin et al. (2010), β-AAs tend to reduce marbling and tenderness. In a study involving lambs, the β-AA clenbuterol, was found to increase the activity of calpastatin, while it decreased the activity of the μ-calpain (McDonagh *et al*., 1999). Koohmaraie (1991) reported that the β-agonist L644,969, increased calpastatin activity in the *biceps femoris* of lambs, while the activities of μ- and m-calpains were unaffected. This suggests that the activity of calpains could possibly be the main effector of reduced protein degradation (Koohmaraie et al. (1991). In a study by Wheeler and Koohmaraie (1992), the authors demonstrated a decrease of 27% in the rates of fractional protein degradation of skeletal muscle myofibrillar protein, as well as an increase in the protein synthesis rates of skeletal muscle myofibrillar protein following 3 weeks of feeding the β-agonist L644,969 in steers. These may collectively be a contributing factor of reduced tenderness in carcasses of animals treated with β-AAs.

Of the proteins that affect meat quality, collagen has received limited attention (Purslow, 2014) and how anabolic steroids and β-AAs affect collagen and its contribution to beef toughness is not well understood. Early research indicated that the solubility of collagen was unaffected by

temperature or time of carcass conditioning (Pierson and Fox, 1976; Chizzolini *et al.,* 1977). This suggests that the structure of collagen does not change at the molecular level during *post-mortem* ageing. However, Stanton and Light (1988, 1990) reported that collagen found in the perimysium is damaged and partly solubilized during *post-mortem* ageing. Judge and Aberle (1982), using a scanning calorimetry showed that the thermal shrinkage temperature of bovine intramuscular collagen decreased within 7 days *post-mortem,* by 7 - 8 °C. Lewis et al. (1991), using a method for measuring the mechanical strength of isolated connective tissue from the perimysium, developed by Lewis and Purslow (1989), showed that the breaking strength of the perimysium of the connective tissue in raw beef decreases during *post-mortem* ageing. Additionally, Nishimura et al. (1995) reported that the integrity of the structure of the IMCT of beef decreases during *post-mortem* ageing. Although the mechanism involved in the weakening of the IMCT remains inconclusive, the IMCT is no longer deemed immutable with *post-mortem* ageing, as previously thought.

According to Bruce and Roy (2019), the use of growth promotants does not appear to influence the solubility of collagen but was shown to increase the concentration and densities of the collagen crosslinks pyridinoline and Ehrlich's Chromogen in bovine ST and GM muscles. While the effect of GP on muscle fibres such as hypertrophy is known, their effect on collagen is not fully understood (Kellermeier *et al.,* 2009; Ebarb *et al*., 2016). In a study by Ebarb et al., (2017), the use of growth promotants neither influenced collagen contents nor the crosslink pyridinoline in beef *longissimus lumborum* steaks. Girard et al., (2011), reported that the use of growth promotants, particularly RH, did not affect the collagen content of the ST and GM muscles. β1-selective agonists such as RH have a less dramatic effect on cattle than β2-agonists (Moody *et al.,* 2000). According to Sissom et al. (2007), β2-adrenergic receptors are the most abundant β-adrenergic receptors in the skeletal muscle of cattle, while the population of β1-adrenergic receptors is minimal (Sillence and Matthews, 1994). As a result, β1-selective agonists do not have much prospect to bind to their receptors to illicit their effect compared to β2-selective agonists (Moody *et al.,* 2000). This may explain why RH in particular has no or limited effect on the collagen content of bovine muscles.

1.7 Genes involved in collagen and collagen crosslink synthesis

How anabolic steroids and β-AA affect collagen structure is largely unknown, although anabolic steroids are widely used in the medical community to increase the rate of connective tissue repair. The synthesis of the various types of collagens can be estimated by measuring the expression of genes associated with their synthesis, and for skeletal muscle would include *COL1A1* and *COL1A2* for the type I collagen α-chains and *COL3A1* for the type III collagen α-chains (Du and McCormick, 2009; Gonzalez *et al*., 2014). The hydroxylation of proline in collagen is also imperative for normal triple-helical molecule formation and stability, and this post-translational modification is affected by prolyl 4-hydroxylase (P4H) in the endoplasmic reticulum. This enzyme catalyzes 4-hydroxyproline formation through the hydroxylation of prolines in -X-Pro-Gly- sequences in collagen molecules and in over 15 other proteins with collagen-like domains (Kivirikko and Pihlajaniemi, 1998; Myllyharju and Kivirikko, 2001). More importantly, it plays a central role in collagen biosynthesis as this hydroxylation ultimately enables the formation of the collagen triple helix (Myllyharju, 2003). P4H had previously been assumed to exist in only one type, with no isoenzymes. This notion was however amended when a second α subunit (α (II) was cloned from humans (Annunen *et al.,* 1997), and mice (Helaakoski *et al.,* 1995).

Another post-translational enzyme that contributes to later reactions that stabilize collagen is lysyl hydroxylase (LH), which activates hydroxylysine formation in X-Lys-Gly- sequence in a reaction that demands the use of Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate (Kivirikko and Pihlajaniemi, 1998). In the later part of the 1990s, three genes that encode isoforms of LH were identified and partly characterized. These genes are *PLOD1–PLOD3* and correspond to the isoforms of lysyl hydroxylase 1 to 3 *(LH1–LH3)* respectively (Valtavaara *et al.,* 1997; Valtavaara *et al.,* 1998). Although the substrate specificities of the above-mentioned isoforms remain inconclusive, there is evidence that LH1 is primarily responsible for the hydroxylation of lysine residues found in the helical domains of fibrillar and non-fibrillar collagens (Kivirikko, 1982). Two alternatively spliced isoforms were identified for the *LH2* gene. These were named LH2a or LH2 (short) and LH2b or LH2 (long). respectively. LH2b consists of an additional 21 amino acids that are encoded by the 63-nucleotide exon 13A. LH2a does not contain these extra amino acids. LH2b is the main isoform of LH2 in most human tissues and seems to be ubiquitously expressed (Yeowell and Walker, 1999). Initial observation of LH2 revealed that its mRNA expression was

closely related to lysine hydroxylation in the telopeptide domains of type I collagen molecule. This led to the proposal of LH2, more specifically LH2b, as a telopeptide LH (Uzawa *et al.,* 1999). The activity of LH2 is regulated by homeodomain transcription factor, which is encoded by the *PITX2* gene, which regulates *PLOD2* (Ghosh, 2007). LH3 on the other hand, can perform activities usually performed by LH, hydroxylysyl galactosyltransferase (GT) and galactosylhydroxylysyl glucosyltransferase *(GGT)* (Wang *et al*., 2007). Its LH activity could potentially be instrumental for collagen types IV and V (Myllyla, 2006).

Another enzyme related to collagen synthesis is lysyl oxidase (LOX), which is secreted by fibroblasts and is responsible for the oxidation of specific peptidyl lysine residues that allows them to crosslink and stabilize the collagen molecules into larger structures (Sambasivarao, 2013). LOX initiates the formation of crosslinks in collagen fibrils through oxidative deamination of specific lysine and hydroxylysine residues to form allysines. The action of LOX on the formation of collagen fibrils is however not entirely understood (Herchenhan *et al.,* 2015).

Increased matrix protein accumulation including collagen types I and III, laminin and fibronectin has been linked to increased circulating levels of transforming growth factor beta 1 (TGFβ-1), which initiates the proliferation of fibroblasts as well as the transformation of fibroblasts into myofibroblasts (Qi *et al*., 2006; Coward *et al.,* 2010). Additionally, TGF-β1 triggers the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), thus ensuring that the level of re-modelling of the extracellular matrix is balanced (Qi *et al.,* 2005). Members of the TGF-β family, particularly TGFβ-1 enhances the expression of *PLOD2* and subsequently, the promotion of pyridinoline crosslinking (van der Slot *et al.,* 2005; Remst *et al.,* 2014). The growth factor IGF-I has also been associated with increased deposition of collagen and proteoglycans, although it has been found to have no influence on the number of crosslinks per collagen molecule (Jenniskens *et al*., 2006). In a study by Huang et al. (2010) using obese sheep, cross-talk was suggested between the mechanisms of IGF-I and TGF-β that collectively reduced the activity of MMPs because obesity is linked to increased levels of circulating and unbound IGF-I (Nam *et al*., 1997). According to Kumar et al. (2004), the use of exogenous hormones such as progesterone and estrogen may result in an increase in circulating IGF-I levels. This may further result in the reduction in the activity of MMPs and collagen

synthesis through reduced phosphorylated levels of IκBα and p65, which ultimately increases the density of mature collagen crosslinks.

MMPs and their endogenous tissue inhibitors (TIMPs) appear to regulate collagen degradation. MMPs are mostly known for their role in the degradation and re-modelling of the ECM, the IMCT included (Murphy, 2010). The different types of TIMPs have different specific actions directed at a range of components of the ECM (Morrison *et al*., 2009). There are four known types of TIMPs (TIMP-1, 2, 3 and 4). TIMP-1, 2 and 4 are however known to have the greatest function against MMPs. These TIMPS inhibit different MMPs according to their location and substrate and regulate the activity of MMPs during tissue remodeling (Baker *et al.,* 2002). TIMPs in some instances may act as activators. For example, they can activate proMMP-2 and pro-MMP9, which are gelatinases responsible for proteolysis of denatured collagens (Troeberg and Nagase, 2007). Aside from their inhibitory effect on MMPs, TIMPs partake in the regulation of other biological processes such as cell growth, repression of angiogenesis, and the induction or reduction of apoptosis (Fassina *et al.*, 2000). TIMPs are important natural inhibitors of MMP-2 and MMP-9 (Brehmer *et al.*, 2003).

The SMAD pathway has been reported by several studies to play a role in the activation of the type I collagen gene (Piek *et al.,* 1999; Massagué and Wotton, 2000). SMADs include a series of proteins that are responsible for the transduction of signals to the nucleus, which is achieved by the downstream actions from the receptors of serine/threonine kinase of the TGF-β family (Piek *et al.,* 1999; Massagué and Wotton, 2000). This SMAD signaling cascade has been shown to play an important role in the production processes of human collagen associated with TGF-β (Lee *et al*., 2006). The receptor regulated SMADs are a part of the SMAD family and are also known as RSMADs. They include SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8, and are downstream effectors of serine-threonine kinase receptors that are activated when bound to TGF-β (Ross and Hill, 2008). Two inhibitor SMADs, (I-SMAD6 and SMAD7) make up the remainder of the SMAD family (Moustakas *et al.*, 2001).

Proteins on cell surfaces such as integrins have been proposed to transmit physical stimuli from the ECM of the tendon to regulate intracellular signaling pathways, as well as gene expression (Khan and Scott, 2009). According to Humphrey et al. (2014), heterodimers of α and β integrins

transduce different mechano-transduction pathways, which is dependent on the isoforms involved, and their interaction with the ECM and cytoplasmic proteins. The β1 integrin is a member of the subfamily of integrins that bind collagens and include α1β1 (*ITGA1*), α2β1 (*ITGA2*), α10β1 (*ITGA10*) and α11β1 (*ITGA11*) (Popova *et al*., 2007; Zeltz *et al.,* 2016). The integrin α1β1 (*ITGA1*) (the α subunit is also referred to as very late antigen-1 VL-1, CD49a) is largely expressed on cells in contact with basement membranes and connective tissue cells (Gardner, 2014). α1β1 (*ITGA1*)

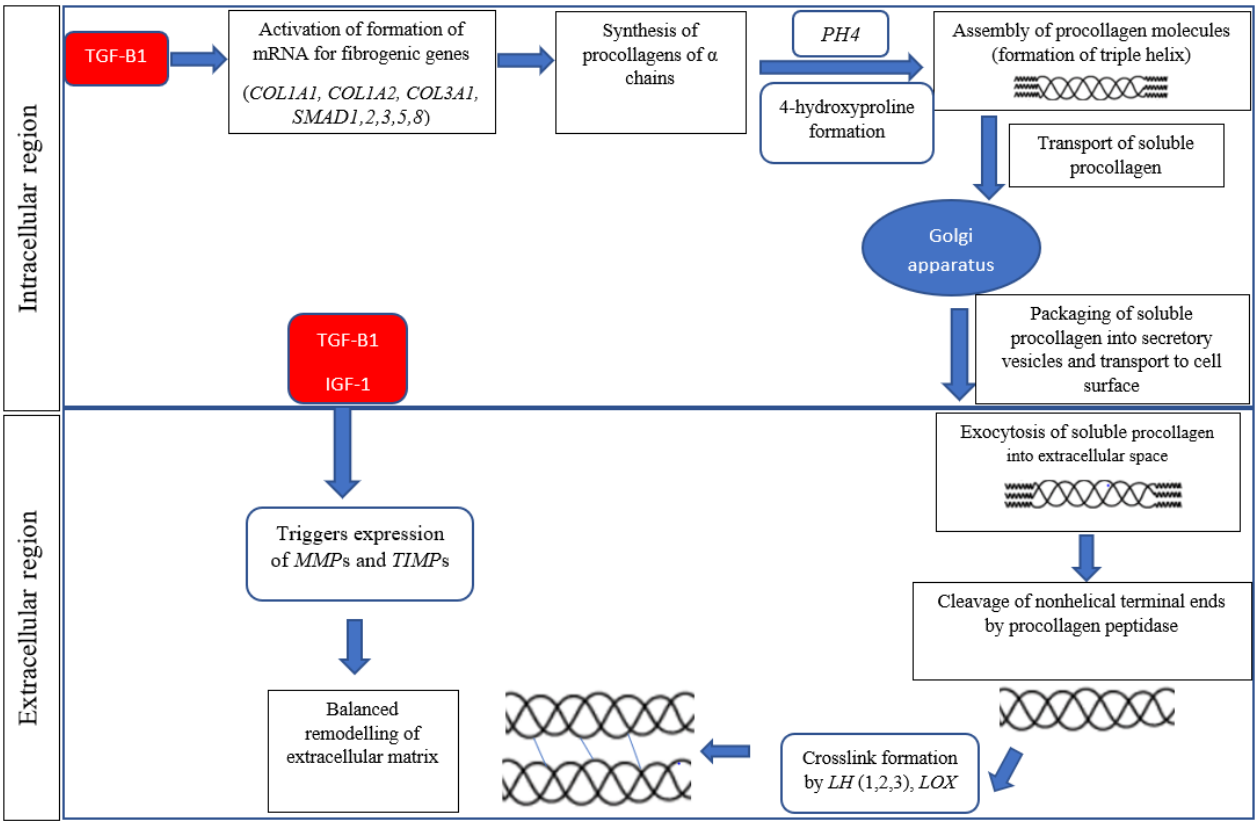

Figure 1.1 Schematic diagram representing the main pathways by which the expression of genes affect collagen synthesis and crosslinking

binds to ligands such as collagen types I, III, IV, IX, XIII, and XVI (Gardner, 2014). When α1β1 (*ITGA1*) binds to ligands, it results in a reduction in the synthesis of collagen, coupled with a reduction in MMP synthesis (Jablonski *et al.,* 2014). It is worth noting that the cytoplasmic tail of

α1 employs complex interactions with the receptors of growth factors to convey some of its effects (Mattila *et al.,* 2008). The integrin α2β1 (*ITGA2*) is expressed extensively in cells that have contact with basement membranes and in cells that have contact with interstitial matrices rich in collagen type I, such as fibroblasts. α2β1 (*ITGA2*) binds to ligands including collagens I, III, IV, V, XI, XVI and XXIII (Kern *et al.,* 1993; Kapyla *et al.*, 2004). Studies on the signaling pathways activated in fibroblasts in 3D collagen matrices have shown that the activation of the signaling and stimulatory effects of p38α MAP kinase (MAPK14) on collagen synthesis is dependent on α2β1 (*ITGA2*) (Ivaska *et al.*, 1999; Ravanti *et al.,* 1999).

1.8 Research Objectives

Numerous studies have focused on the contribution of muscle fibres to beef tenderness. On the contrary, the contribution of collagen and its associated crosslinks to beef tenderness has not received due attention, particularly at the gene expression level. In a study by Fontes et al. (2021), the synthesis and metabolism of *longissimus thoracis* intramuscular collagen in heifers and cull-cows given feed that was high in energy for equal number of days (150) were investigated. This was done by the comparison of the deposition of intramuscular collagen and the expression of markers of remodeling of collagen between the two groups of cattle, before slaughter. Results from collagen content analysis showed no significant difference for total collagen between the two cattle groups. The authors reported a difference in the collagen solubility among the two groups where it was higher in heifers, which was however not associated with the activity of LOX as it was similar among the two groups. Results from mRNA abundance using RT-qPCR showed no differences in the mRNA expression of genes implicated in collagen synthesis which included *COL1A1, MMP2, MMP9* and *TIMP2.* For other genes equally implicated in collagen synthesis including *COL3A1, TIMP1* and *TIMP3,* their mRNA expression was higher in cull-cows than heifers, signifying an increase in collagen synthesis in the later than in the former group. A study by Chen et al. (2011) explored the global gene expression profiling in the identification of genes that are differentially expressed in relation to feed efficiency in beef cattle, using bovine microarrays. The authors reported that *COL1A1* and *COL3A1* were upregulated in low RFI Angus cattle. This was however through an interaction with platelet-derived growth factor. Chen et al. (2019) using RNA-sequencing identified differently expressed genes in the *longissimus* muscle of crossbred cattle which influenced marbling, an important beef quality trait. Results from the study

showed that myogenic genes such as *MyoG,* was upregulated in the group categorized as low marbling. Their results also showed that fibrogenic genes including *COL11A2* and *COL22A1* were upregulated in the grouped categorized as high marbling, while *COL14A1, COL16A1* and *COL1A2* were downregulated in the same group. There is however a knowledge gap between the expression of gene and how they directly influence meat quality. The effect of mRNA abundance of gene associated with growth and meat quality is an area of study that warrants further research as gene expression is known to affect biological characteristics. This thesis focused on the effect of selection for RFI, and the utilization of two (2) growth promotants, ractopamine hydrochloride (RH) and hormonal implants/steroids, individually and in combination on steer growth performance and meat quality. Special attention was focused on their effect on collagen and collagen crosslinks and their contribution to beef tenderness. The thesis also further examined mRNA expression levels of proteins involved in the synthesis of collagen and collagen crosslinks, as they are reported to be responsible for the background toughness of beef, in relation to RFI status and growth promotant utilization. To achieve this goal, the thesis was divided into three (3) sections with different objectives, to ultimately aid in resolving the challenge of beef toughness and inconsistencies in beef tenderness. My main objectives for the thesis are as follows:

1. To determine the effect of RFI status, steroids and RH, and their interaction on growth performance, carcass, and meat quality characteristics of crossbred Angus steers.
2. To relate growth performance measures with meat quality characteristics.
3. To elucidate how collagen and collagen crosslink characteristics are modified, if at all, in response to selection for RFI and growth promotant treatment, and *post-mortem* ageing and relate them with steer growth performance and meat quality characteristics.
4. To quantify the level of expression of genes associated with proteins involved in collagen and collagen crosslink synthesis, and muscle *post-mortem* proteolysis.
5. To relate gene expression levels with growth performance, meat quality characteristics and collagen solubility.

I hypothesized that:

1. The likelihood of a decrease in beef tenderness occurs through an interaction between selection for low RFI in beef cattle with steroid and/or β-adrenergic agonists use.

2. The circulatory levels of the growth factors IGF-I and TGF-β1 increase through the modulation of cell activity caused by the use of steroids.
3. Selection for low RFI does not impact collagen solubility characteristics.
4. The use of steroids and β-adrenergic agonists increase total intramuscular collagen concentration and the densities of the pyridinoline and Ehrlich's chromogen crosslinks through the increased activity of TGF-β1 and its effect on the SMAD pathway, and activation of adenyl cyclase respectively.
5. *Post-mortem* ageing increases the heat solubility of the intramuscular collagen through the weakening of the intramuscular connective tissue.
6. Selection for low RFI status decreases the expression levels of genes associated with proteins involved in collagen synthesis degradation (*ITGB1, ITGA11, SMAD2, SMAD3, FN1, FGF2, COL1A1, COL3A1*), and increase the expression level of genes associated with collagen cross-link synthesis (*LOX, LH1, LH3*).
7. Beef tenderness is decreased through selection for low RFI, and use of steroids and β-adrenergic agonists which increase the expression level of calpastatin (*CAST*), and reduce the expression level of calpains (*CAPN1*).
8. Steroid and β-adrenergic agonists increase expression levels of genes associated with proteins involved in collagen and cross-link synthesis (*ITGB1, ITGA11, SMAD2, SMAD3, LOX, LH1, LH3, FN1, FGF2, COL1A1, COL3A1*) through the increase in mRNA production.

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Chapter 2. Relationships between residual feed intake, the use of growth promotants, and the growth and meat quality characteristics of crossbred Angus steers

2.1 Introduction

The feed conversion efficiency (FE) achieved in the beef industry using common growth promoting technologies is important in sustaining the financial (Duckett and Pratt, 2014) and environmental viability of the industry (Capper, 2011). The two most common types of growth promotants available to beef producers are anabolic steroid hormone implants and β-adrenergic agonists (β-AA). Combined trenbolone acetate (TBA) and estradiol 17β implants have been observed to improve growth rate and FE by 20% and 15% respectively (Bartle *et al.*, 1992; Johnson *et al.,* 1996a), while implants comprised of TBA and estradiol (E_2) increase performance and hence profit, in feedlot cattle (Duckett and Pratt, 2014). Ractopamine hydrochloride (RH) is at present the sole β-AA available to beef producers. According to Lean et al. (2014), RH increased the average daily gain (ADG) of beef steers by 0.19 kg and Warner-Bratzler Shear Force (WBSF) by 0.2 kg, although Quinn et al. (2008) found no effect of RH on WBSF values. Similar results have been obtained for the effects of steroids on beef quality, with steroids increasing WBSF measured in the early (2-7 days) *post-mortem* period (Ebarb *et al*., 2017). This increase in beef toughness with the use of steroid implants in beef cattle has been associated with increased activity of calpastatin (Strydom *et al*., 2009), which inhibits the calpain enzymes considered to be responsible for proteolysis of myofibrillar proteins during *post-mortem* ageing. The use of steroid implants in beef cattle has also been associated with increased cross-sectional area of muscle fibres (Ebarb *et al.,* 2016) and increased hydroxylysylpyridinoline (HP) cross-link formation in collagen (Roy *et al.,* 2015), both of which may contribute to increased WBSF values.

Feed efficiency in beef cattle has also been achieved through selection for reduced feed intake at a constant rate of growth (Arthur and Herd, 2008). Residual feed intake (RFI) as an indicator of FE in growing cattle does not depend on production traits being used to calculate expected feed intake. This allows for the selection of this trait without antagonizing mature body weight and growth rate of breeding stock (Koch *et al.*, 1963). Selection for low RFI however, maybe accompanied by reduced deposition of intramuscular fat, which has the potential to negatively impact meat tenderness (Richardson *et al*., 2001; Nascimento *et al.*, 2016). Other research suggests that RFI has little effect on meat quality when cattle are not treated with growth-

promoting technologies (Baker *et al.,* 2006), and is more affected by breed than RFI status (Jiu *et al.,* 2020). Selection for low RFI may interact with growth promoting technologies to decrease beef tenderness.

2.2 Objective

The objective of this study was to examine how RFI status interacts with the RH and steroid implants on beef cattle growth, carcass and meat quality.

2.3 Materials and methods

2.3.1 Animal care and management

The study was approved by the Research Ethics Committee at the University of Alberta, Edmonton, Canada (AUP 00000777). Cattle were cared for in accordance with guidelines provided by the Canadian Council of Animal Care (CCAC). Angus crossbred bull calves used in this study were produced as described by Jiu et al. (2020), with a hybrid dam line (Kinsella composite) crossed with Angus bulls. The hybrid dam line was approximately 33% each of Angus and Charolais and 20% Galloway, with the remaining 14% a mixture of other beef breeds detailed by Goonewardene et al. (2003) and Nkrumah et al. (2007). All calves were born and raised and the experiment conducted at the Roy Berg Kinsella Research Ranch (Kinsella, Alberta). All calves were born in April or May of 2015, and were uniquely identified using ear tags. Bull calves were castrated using scrotal banding at birth and pastured with their dams at the ranch until weaning at approximately 6 months of age, when they were moved to their treatment pens.

2.3.2 Experimental design and treatments

Forty-eight crossbred Angus steers bred either from a line of cattle selected for low RFI or from one without selection for RFI (control) at the University of Alberta Roy Berg Kinsella Ranch were stratified by weaning weight within RFI status. The steers were then randomly assigned to one of four treatments within RFI status in a 2 × 2 × 2 factorial design study. Treatments consisted of no implant and no RH (Control, n =12), implant only (IMP, n = 12), RH only (RH, n = 12), and IMP and RH (n = 12). Steers randomly assigned to treatment with implants were first implanted

with Component E-S (200 mg progesterone, 20 mg estradiol benzoate and 29 mg tylosin tartrate, Elanco Animal Health, Eli Lily Co., Greenfield, Indiana) at a mean age of 328.25 ± 8.60 days and at a live weight of 383.19 ± 2.90 kg (± standard error of the mean). Steers were implanted with a second steroidal growth promotant, Component TE-S (120 mg trenbolone acetate, 24 mg estradiol and 29 mg tylosin tartrate) 90 days after the first implant.

2.3.3 Diet

At weaning, at about 6 months of age, steers were gradually alimented onto a primarily forage background diet (Table 2.1) at about 11 months of age. Steers were gradually alimented onto a finishing diet (Table 2.1) upon application of the first steroid and remained on this diet for the duration of the study, which was about 7 months. During the last 28 days prior to slaughter, steers were fed either a targeted 200 mg RH/head/day (top-dressed, Optaflexx, Elanco Animal Health, Eli Lily Co., Greenfield, Indiana) or not (controls) while they remained on the finishing diet. Steers were allocated to one pen per treatment. Although RH has no withdrawal period, steers receiving RH were fed the control diet a day before slaughter. Slaughter occurred serially over a period of 6 weeks.

Table 2.1 Composition of weaning and finishing diets (% as fed)

Feed ingredients (%)	**Weaning diet**	**Finishing diet**
Barley silage	72	27
Barley	0	61
Oats	21	0
Canola meal	4	8
Rumensin/mineral premix	3	4

2.3.4 Growth performance data

Feed intake was recorded by a Growsafe System (Airdrie, AB, Canada) daily from 51 days after the initial implantation until the day of slaughter. Measuring feed intake from day 0 of study was not possible due to use of the facility by other researchers. Feed intake data was used to calculate the amount of RH consumed, average daily feed intake (ADFI), ADG and RFI. Live cattle body weight (BW) was measured and recorded monthly or at handling until they reached approximately 8 mm back fat (approximately 5 months), and then weekly during the final 28 days prior to slaughter when steers received RH. Consequently, FE was calculated during the first implant phase after the steers entered the Growsafe System and during the RH phase using the equation below.

$$\text{Feed conversion efficiency (FCE)} = \frac{\text{Weight gain (kg) during the period}}{\text{Feed intake (as fed, kg) during the period}}$$

The amount of RH consumed by each steer was calculated as:

RH consumption (mg/head/day) = [ADFI (kg/day) × 0.04] × 224.7 (mg RH /kg premix)

Where 0.04 indicates the 4% inclusion rate of the vitamin and mineral mixture feedlot supplement that contained RH (224.7 mg RH/kg of premix as fed).

RFI was calculated as described Jiu et al. (2020). Steer RFI values were calculated as the difference between actual and predicted dry matter intake (DMI) for each steer within a data set that included all calves born that year. Predicted DMI was calculated using ADG and metabolic body weight (MWT) calculated as midpoint $BW^{0.75}$, where midpoint BW was the sum of initial BW and ADG multiplied by half of the days on test (Equation 1). Residual feed intake was adjusted for back fat thickness (Equation 2) to negate effects on RFI due to differences in physiological age (Basarab *et al*., 2011) with DMI adjusted for ultrasound back fat thickness used to calculate RFIf. The models used to predict expected DMI and expected DMI adjusted for ultrasound back fat thickness were described by Mao et al. (2013) and were:

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 MWT_i + e_i \qquad (1)$$

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 MWT_i + \beta 3 FUFAT_i + e_i \quad (2)$$

Where β_0 is the intercept; β_1 β_2 and $\beta 3$ are the coefficients on ADG, MWT and FUFAT, respectively; e_i is the residual.

2.3.5 Blood sampling

Blood was collected from each steer by jugular venipuncture into 10 mL sterile Vacutainers™ containing ethylenediaminetetraacetic acid (EDTA) (Vacutainer, BD Diagnostics, Franklin Lakes, NJ USA) 30 days after the second implant (day 121 of the study) into serum separating tubes. Two Vacutainers™ of blood were collected from each steer and immediately stored on ice until processed. Plasma was obtained from the blood samples by centrifugation at 1,500 *g* for 10 minutes at 4 °C, collected in separate sterile tubes and stored at -80 °C for the quantification of the concentrations of IGF-1 and its binding protein (IGFBP-3).

2.3.6 Quantification of growth factors IGF-1 and IGFBP-3

Commercial sandwich enzyme immunoassay kits specific for quantitative measurement of IGF-1 (SEA050Bo bovine IGF-1 ELISA) and IGFBP-3 (SEA054Bo bovine IGFBP-3 ELISA) (Cloud-Clone Corp., Katy, Texas, USA Sandwich Assays) were used (Das and Seth, 2017; Lakhani *et al.,* 2020). Procedures for the assays were as specified by the manufacturer. A standard curve for each growth factor was derived by regression of known standard concentrations on to their optical densities (O.D). The O.D of the standards were used to ensure that the intra-assay coefficient of variation was less than 10 (CV < 10). Calculation of the final IGF-1 and IGFBP-3 plasma concentrations considered sample dilution, and the means of duplicate assays were used for statistical analysis.

2.3.7 Muscle sampling

Steers were slaughtered according to provincial regulations at a provincially-registered slaughter plant, in blocks of 8 steers over 6 weeks, with each treatment represented in each block until all steers in that treatment were processed. Hot carcass weights were recorded for each carcass and the carcasses chilled for 48 h at 4 ± 1 °C. The carcasses were then fabricated and the *gluteus medius* (GM) and *semimembranosus* (SM) muscles removed from the right side and individually packaged under vacuum. The muscles were then transported on ice to the laboratory and stored at

4 ± 1 °C. At 72 h (3 days) *post-mortem*, each muscle was weighed and then divided into two halves: caudal and cephalad. Muscle halves were assigned randomly to 3 or 12 days *post- mortem* (dpm) ageing and balanced for location within the muscle across slaughters. Meat quality analyses were performed immediately on the half assigned to 3 dpm ageing, while the other half was packaged under vacuum in polyethylene bags (Thermo Scientific, Waltham, Massachusetts) and stored at 4 ± 1 °C for 9 additional days, and then analyzed for meat quality at 12 dpm.

Following each ageing period, muscle halves were sectioned into steaks (Figures 1 and 2). For the GM muscle, 2.5 cm thick steaks for meat quality analyses were removed sequentially toward each end of the muscle (Figure 2.1b). The first steak (p) was used for sensory analysis in another study, the second steak (q) for drip loss, cooking loss and WBSF measurements, and the third steak (r) for intramuscular connective tissue (IMCT) isolation in another study. The fourth steak (s) was first used for the measurement of pH and temperature, after which a fresh surface was exposed to allow for blooming for color measurement. After color measurement, the epimysium of the steak was trimmed and discarded, and the steak cut into small cubes, which were weighed into a pre-weighed aluminium tray and frozen at -20 °C until lyophilized in preparation for proximate analyses (Figure 2.1b). Proximate analysis was performed on muscles aged for 3 days only.

For the SM muscle, approximately 2.5 cm thick steaks were obtained from either the caudal or cephalic ends sequentially toward the center of the muscle (Figure 2.2b). The first steak (either caudal or cephalic (p) was used for sensory analyses in another study, the second steak (q) was used for drip loss, cooking loss and WBSF measurement. The third steak (r) was used for intramuscular connective tissue (IMCT) isolation for another study, and the fourth steak (s) for the measurements of intramuscular pH and temperature, color and proximate analyses. Again, proximate analysis was performed on muscles aged for 3 days only. Just prior to removal of the fourth steak, the intramuscular pH and temperature were measured at three separate locations along the cut line between the 3rd and 4th steak for both muscles. For both muscles, steaks dedicated to IMCT isolation were vacuum-packed and stored at -20 °C until further processing.

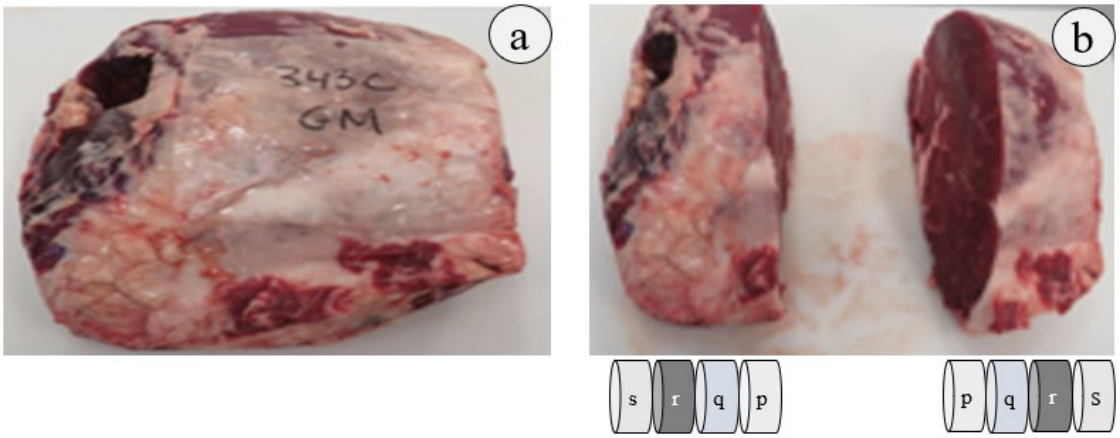

Figure 2.1 Sampling of steaks from the *gluteus medius* (GM) muscle of cross-bred Angus steers at 3 and 12 days *post-mortem* (dpm).

a) Whole GM muscle; b) Halves of GM muscle and steaks (p = sensory analysis for another study; q = drip loss, cooking loss, WBSF; r = isolation of IMCT; s = pH, temperature during pH measurement, color, lyophilized for proximate analyses) were used for different parameters estimation either at 3 dpm or 12 dpm.

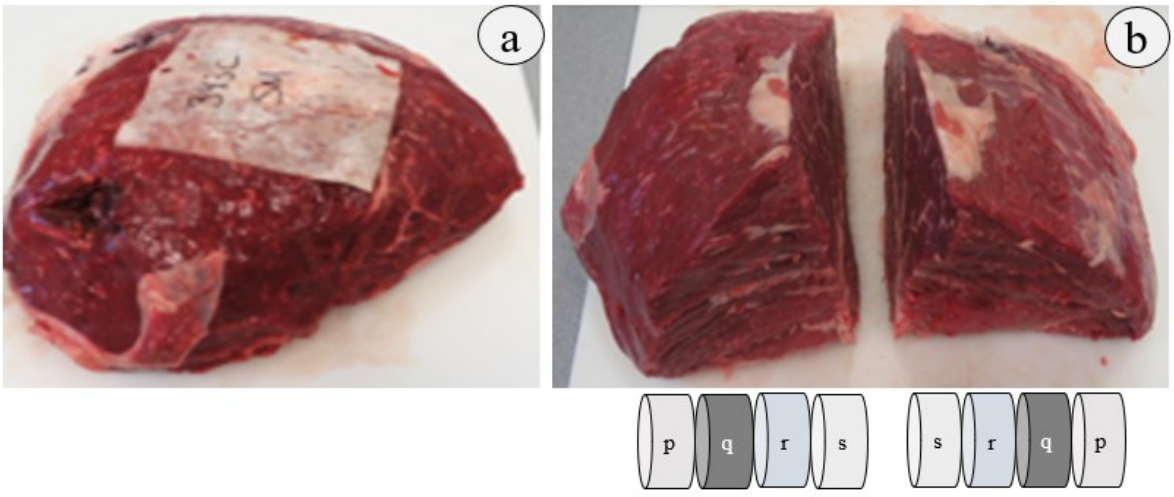

Figure 2.2 Sampling of steaks from the *semimembranosus* (SM) muscle of cross-bred Angus steers at 3 and 12 days *post-mortem* (dpm).

a) Whole SM muscle; b) Halves of SM muscle and steaks (p = sensory analysis for another study; q = drip loss, cooking loss, WBSF; r = isolation of IMCT; s = pH, temperature during pH measurement, color, lyophilized for proximate analyses) were used for different parameters estimation either at 3 dpm or 12 dpm.

2.4 Meat quality analyses

2.4.1 Intramuscular pH and temperature

Before muscles were fabricated into steaks, the intramuscular temperature and pH were recorded on each muscle between the 3rd and 4th steak cut line using a Fisher Scientific Accumet Waterproof AP71 pH/mV/Temperature meter (Fisher Scientific, Mississauga, ON, Canada). The pH meter was fitted with a pH electrode (Cat No. 655-500-30, FC210B, Canada-wide Scientific, Ottawa, ON), and a temperature probe. The pH electrode was calibrated to a pH of 4.0, 7.0 and 10.0 respectively, using commercial standards (Fisher Scientific, Ottawa, ON, Canada). The pH and temperature were recorded simultaneously in triplicate and the average of the three readings was used for statistical analysis.

2.4.2 Color, Drip and Purge losses

Following removal of the first and second steaks for both GM and SM muscles, the cut surface of the steak was exposed to air for 20 min. to bloom at room temperature. Color was measured over three readings, each recorded at a different location using a Minolta Chroma Meter (CR-400, Konica Minolta, Osaka, Japan). The Chroma Meter was calibrated prior to measurement using a white tile supplied by the manufacturer and had an 8 mm aperture operating with the D65 illuminant, with the observer angle set at 2°. The mean of the three measurements was used for statistical analysis.

For purge loss, the steak previously packaged under vacuum was weighed after the respective days of ageing together with its purge in its package (A). The muscle was then removed from the bag, blotted dry with paper towels and weighed together with the dried package (B). Purge loss (%) was calculated as [(A-B)/A] × 100. Drip loss was determined using the bag method (Honikel, 1998). In this method, drip loss was measured as the weight loss during the suspension of about 100 g of muscle sample (approximately 2.5 cm × 7 cm × 5 cm) on a steel hook in an inflated plastic bag for 24 h at 4 °C. The final muscle weight was recorded, and drip loss was expressed as percentage relative to the initial weight of the muscle [(Initial muscle weight – Final muscle weight)/Initial muscle weight] × 100].

2.4.3 Cooking loss and Warner-Bratzler shear force

Steaks weighing 188 ± 5 g were placed in polypropylene bags after having a temperature probe (Tinytag View 2 TV-2040; Gemini Data Loggers Ltd. West Sussex, UK) inserted into their geometric centre. Steaks were cooked in a water bath set at 73 °C until the steaks reached an internal temperature of 71 °C. The bags were then removed from the water bath and immersed in ice water to discontinue the cooking process, after which they were stored for 24 h at 4 °C. After 24 h, steaks were removed from refrigeration and allowed to equilibrate to room temperature before being blotted dry and weighed for cooking loss as percentage with the equation [(Muscle weight before cooking – cooked muscle weight after refrigeration)/ Muscle weight before cooking) × 100].

Warner-Bratzler Shear Force was measured using the American Science Association (AMSA) instrumental measures of tenderness and textural properties (AMSA, 2012; 2016). A metal corer, 1.27 cm in internal diameter, was used to obtain six cores from the steaks after cooking loss measurements. Cores were removed parallel to the muscle fibres, avoiding large deposits of fat and connective tissue. A material testing machine (Lloyd Instrument LRX plus, AMETEK, Digital Measurement Metrology Inc. Brampton, ON) fitted with a Warner-Bratzler-like V-notch blade at 60° angle was used to estimate WBSF of the cooked meat samples. Each core was sheared through its middle perpendicular to the muscle fibre direction at a speed of 200 mm/min. For each sample, peak WBSF values of the six cores were recorded and averaged, and the means were used for statistical analyses.

2.4.4 Proximate analysis

Samples stored at -20 °C were lyophilized in a VirTis freeze dryer (SP Industries/SP Scientific, Warminster, PA, USA). The lyophilized samples were pulverized using a laboratory blender to which three pellets of dry ice were added. The pulverized samples were then stored in Nasco Whirl-Pak (Zefon International, Inc. Ocala, FL) bags at -20 °C until further analysis. Crude fat was analyzed using duplicate 2 ± 0.0030 g pulverized sample. The extraction of crude fat was performed following the method by the Association of Official Analytical Chemists (AOAC, 1995: Method 960.39) using petroleum ether in a Foss Soxtec 2050 (Foss Analytical, Hillerod, Denmark). For crude protein analyses, 100 ± 5 mg of pulverized muscle samples were analyzed in

duplicate for nitrogen content (Method 992.15; AOAC, 1995) using a TruSpec Carbon/Nitrogen Determinator (LECO Corporation, St. Joseph, MI, USA). Standardization and calibration were performed with a certified rye standard and a certified ethylenediaminetetraacetic acid (EDTA) standard for nitrogen determination. To obtain the total moisture of the fresh meat sample before being freeze-dried, 2 ± 0.005 g of lyophilized sample were heated in a glass vial at 100 °C in an oven for 18 h. The oven dried samples were then weighed to calculate total moisture in the raw meat, and further reduced to ash at 490 °C for 22 h in a muffle furnace (Kejia Furnace Co. Ltd. Henan, China) and weighed to calculate the ash percentage.

2.4.5 Monthly average temperatures in feedlot

Minimum, maximum and average monthly temperatures obtained for the Kinsella Research Station from the Alberta Climate Information Service, an Alberta Government weather station electronic archive (https://acis.alberta.ca/weather-data-viewer.jsp). Temperatures were recorded for the months of the study (April to November 2016).

2.5 Statistical analyses

Statistical analyses were performed using R Studio (Version 3.5.1). Analysis of covariance (ANCOVA) was used to test the effects of RFI and steroid applications and where appropriate ractopamine and their interactions on growth characteristics. Initial weight before steroid treatment was used as a covariate in the analyses. Data for these measurements were analyzed as either a 2 × 2 factorial with RFI and steroid application and their interaction serving as fixed effects if the measurement was performed prior to ractopamine supplementation. Otherwise, data were analyzed as 2 × 2 × 2 factorials, where RFI, steroid and ractopamine and their interactions served as fixed sources of variation. Data for steer growth during the first and second steroid implant phases were analysed as repeated measures with steroid, days on study and their interaction as fixed effects, and animal as a random source of variation.

Analysis of meat quality data was performed using a split-plot design with RFI, steroid and ractopamine hydrochloride and their interactions as fixed effects at the animal (whole plot) level and days of *post-mortem* ageing (DOA) and its interactions as fixed effects at the muscle (split-plot) level. Data were blocked by kill (slaughter day) and animal was a random source of variation. Analysis of hot carcass weight (HCW) data was performed as a mixed effect model, with RFI,

steroid and RH, and their interaction as the fixed effects. Data was blocked by kill (slaughter day) and animal was a random source of variation. A simple ANOVA was used to analyze results from the proximate analyses. For significant ($P \leq 0.05$) models, the means were compared using planned least squares mean comparisons, with Tukey's method used for least squares mean comparisons between interaction means. Pearson's correlation coefficient analysis was performed to identify linear relationships between live weight characteristics and meat quality traits using the rcorr function in the Harrel Miscellaneous Package (Hmisc) in R Studio. A Bonferroni correction was applied by dividing the α- value of 0.05 by the number of variables (Rice, 1989). As such, correlations were considered significant at P values ≤ 0.001. Correlations ranging from 0.20 to 0.39 were considered as weak, values from 0.40 to 0.59 were considered as moderate, values from 0.60 to 0.79 were considered as strong, and values from 0.80 to 1 were considered as very strong. Regression analysis was performed to show relationships between feed intake and body weight gain during the RH supplementation phase. Relationships were considered significant at $P \leq 0.05$.

2.6 Results

2.6.1 Live animal growth and carcass characteristics

One animal (of low RFI status, implanted and RH-supplemented) was removed from the study prior to slaughter due to laminitis. The growth and growth performance data for this steer were excluded from all analyses. One GM muscle from a steer of low RFI status, not implanted but supplemented with ractopamine hydrochloride (RH), was not included in the data analyses as it was damaged during excision from the carcass.

Live animal growth characteristics were considered before the administration of RH for both first and second implantation phases (separately and together), during the administration of RH, and for the full study. Steer weight at initiation of the study (study day 0) was not different between the treatments ($P = 0.0882$) (Table 2.2). Average daily gain calculated for the first implant phase (days 0 to 90) was higher in steers treated with steroids than in steers not treated with steroids ($P < 0.0001$) (Table 2.2) leading to increased mean body weights in steroid treated cattle (Figure 2.3). Average daily feed intake (ADFI) from day 51 to 90 was greater in implanted steers than in non-implanted steers during the first implantation phase ($P = 0.0105$) and steers treated with steroids ended with a higher body weight than untreated steers ($P < 0.0001$) (Table 2.2) (Figure

Table 2.2 Least squares means (with standard errors of the means) of steer growth and carcass characteristics as affected by RFI status and steroid treatment during the first (study day 0 to 90) and second (study day 91 to 148) steroid implant periods.

Measurement	RFI		Steroid		P values[1]		
	Low	High	Steroid	Control	RFI	Steroid	RFI × Steroid
n[2]	26	21	23	24			
First implant (study day 0 to 90)							
Initial body weight (study day 0), kg	379.00 (3.80)	389.00 (4.22)	384 (4.15)	383 (4.05)	0.0882	0.9106	0.6494
Average daily gain (study day 0 to 90), kg	1.35 (0.03)	1.31 (0.03)	1.50 (0.03)[a]	1.16 (0.03)[b]	0.5150	<0.0001	0.8030
Average daily gain (study day 51 to 90), kg	1.23 (0.06)	1.19 (0.07)	1.27 (0.06)	1.15 (0.06)	0.6546	0.1831	0.8569
Average daily feed intake (study day 51 to 90), kg/head	15.10 (0.23)	15.50 (0.25)	15.80 (0.24)[a]	14.90 (0.23)[b]	0.2075	0.0105	0.5574
Feed conversion efficiency (study day 51 to 90) (gain: feed intake)	0.082 (0.004)	0.078 (0.004)	0.081 (0.004)	0.078 (0.004)	0.4190	0.5810	0.9852
Second implant period (study day 91 to 148)							
Body weight at the end of first implant and start of second implant (kg) (day 91)	501.00 (3.65)	503.00 (4.20)	515.00 (3.98)[a]	489.00 (3.76)[b]	0.7978	<0.0001	0.4081
Average daily gain, kg (study day 91 to 148)	1.75 (0.05)[a]	1.57 (0.05)[b]	1.88 (0.06)[a]	1.45 (0.05)[b]	0.0334	<0.0001	0.0236
Average daily feed intake, kg/head (study day 91 to 148)	16.60 (0.25)	16.60 (0.28)	17.10 (0.27)[a]	16.10 (0.26)[b]	0.8790	0.0146	0.0464
Feed conversion efficiency (gain: feed intake) (study day 91 to 148)	0.104 (0.003)[a]	0.095 (0.003)[b]	0.109 (0.003)[a]	0.090 (0.003)[b]	0.0273	<0.0001	0.1487
Growth factors in blood plasma (study day 121)							
Insulin-like Growth Factor-1 (IGF-1, ng/mL plasma)	264.00 (16.30)	281.00 (19.60)	253.00 (18.10)	291.00 (18.10)	0.5039	0.1432	0.0532
Insulin-like Growth Factor Binding Protein-3 (IGFBP3, ng/mL plasma)	1399.00 (72.40)	1396.00 (79.70)	1398.00 (78.20)	1397.00 (74.00)	0.9774	0.9938	0.9471
Cumulative steroid implant effects (day 0 to 148)							
RFI Value	0.160 (0.12)[a]	0.568 (0.14)[b]	0.280 (0.13)	0.466 (0.13)	0.0250	0.3169	0.6872

Average daily gain, kg	1.50 (0.02)	1.44 (0.02)	1.65 (0.02)[a]	1.29 (0.02)[b]	0.0823	<0.0001	0.2418
Cumulative steroid implant effects (day 51 to 148)							
Average daily gain, kg	1.59 (0.03)	1.51 (0.04)	1.71 (0.03)[a]	1.39 (0.03)[b]	0.1103	<0.0001	0.1134
Average daily feed intake, kg/head	15.90 (0.23)	16.10 (0.26)	16.50 (0.25)[a]	15.60 (0.24)[b]	0.6319	0.0150	0.1196
Feed conversion efficiency (gain: feed intake)	0.099 (0.002)[a]	0.093 (0.002)[b]	0.103 (0.002)[a]	0.089 (0.002)[b]	0.0159	<0.0001	0.2593
Body weight after second implantation (day 148), kg	632.00 (2.93)[a]	622.00 (3.27)[b]	657.00 (3.09)[a]	597.00 (3.01)[b]	0.0391	<0.0001	0.0792

[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.
[1] Probability of the F test, with significance at $P \leq 0.05$.
[2] Steer removed from study was of low RFI status, implanted and supplemented with ractopamine hydrochloride.

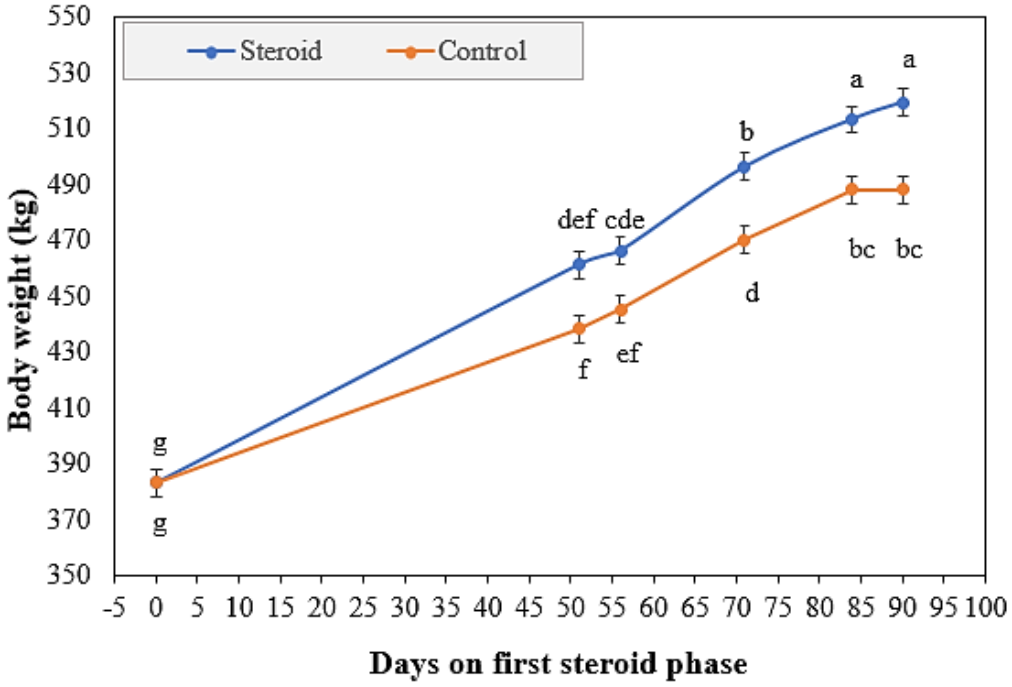

Figure 2.3 Mean body weight of steers as affected by application of the first steroid (Component E-S) in the first 90 days of the study. Treatment means with different letters in the same period of study are significantly different due to treatment ($P < 0.05$).

2.3). Feed conversion efficiency (gain to feed intake) and ADG during the period in which the steers were in the Growsafe System during the first steroid implant period (experimental period from day 51 to 90) did not differ due to treatment ($P > 0.05$) (Table 2.2).

During the second steroid implant period (day 91 to 148), there was a significant interaction between steroid implant and RFI treatments for ADG ($P = 0.0236$) (Table 2.2) that indicated that when treated with steroids low RFI steers had a greater mean ADG than all other treatments (Figure 2.4). High RFI steers showed no increase in ADG with steroid application and had mean values that were not different to those of low RFI steers that did not receive steroids (Figure 2.4). This interaction was accompanied by an interaction between RFI and steroid treatment for ADFI during the second implant phase ($P = 0.0463$) where low RFI steers that received steroids had a mean ADFI greater than that of steers from the other treatments (Figure 2.5). Also, during the second steroid implant phase, FE was greater in low RFI steers than in high RFI steers ($P = 0.0273$), and

greater in steroid implanted steers compared to non-implanted steers ($P < 0.0001$) (Table 2.2). An increased live body weight of steers due to the first implant phase was obvious at the onset of the second phase, and it persisted and was maintained until the end of the phase (Table 2.2) (Figure 2.6).

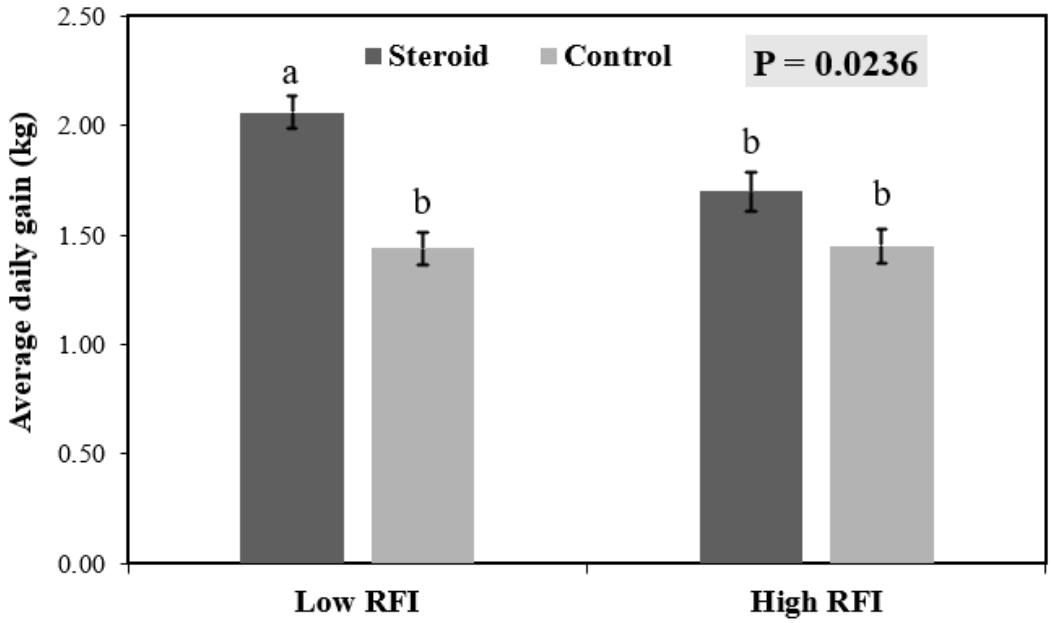

Figure 2.4 Interaction between RFI and steroid treatment on mean average daily gain (kg/ day) of crossbred Angus steers during the second implantation phase ($P = 0.0236$) (Low RFI and implanted, n = 13; Low RFI and not implanted, n = 13; High RFI and implanted, n =10; High RFI and not implanted, n = 11). Treatment interaction means with different letters (a, b) differ according to least square mean differences at $P < 0.05$.

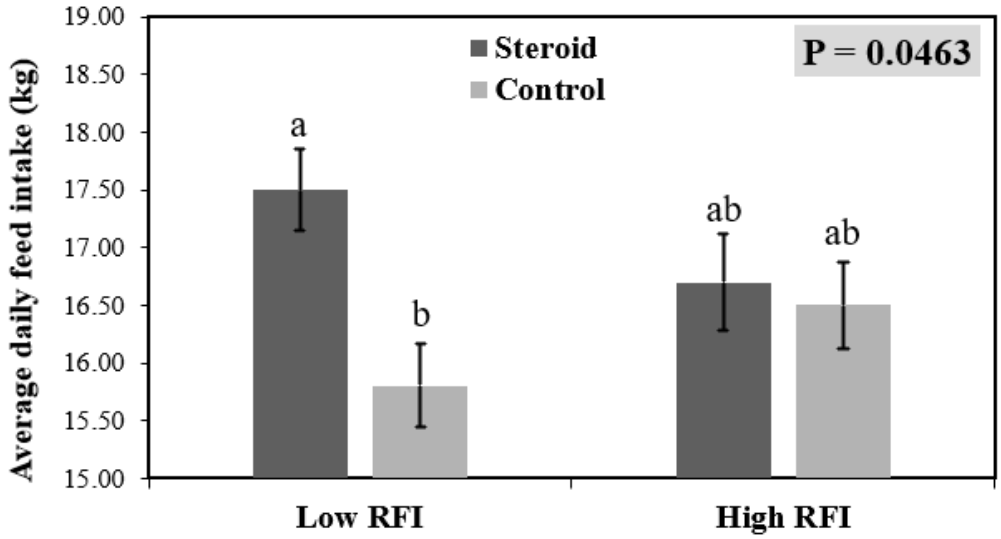

Figure 2.5 Interaction between RFI status and steroid treatment on average daily feed intake (kg) of crossbred Angus steers during the second implantation phase (*P* = 0.0463) (Low RFI and implanted, n = 13; Low RFI and not implanted, n = 13; High RFI and implanted, n = 10; High RFI and not implanted, n = 11). Treatment interaction means with different letters (a, b) differ according to least square mean differences at $P < 0.05$.

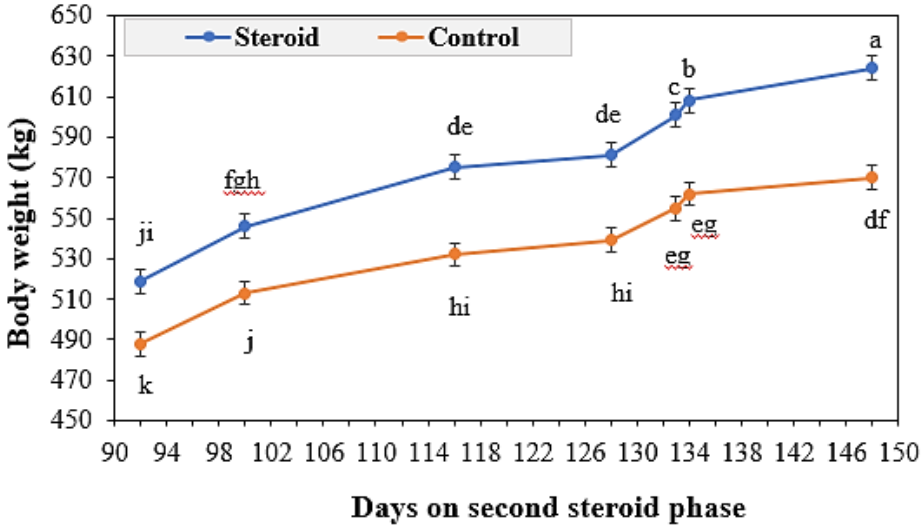

Figure 2.6 Mean body weight of steers as affected by application of the second steroid (Component TE-S) from day 91 to day 148 of the study. Treatment means with different letters in the same period of study are significantly different due to treatment ($P < 0.05$).

Mean circulating IGF-1 was similar in implanted and non-implanted steers ($P = 0.1432$) and was also unaffected by RFI status (Table 2.2). Similarly, IGF-1 binding protein 3 concentration was unaffected by RFI status or steroid treatment (Table 2.2). When data for the first and second implant periods were combined, mean ADG (day 0 to 148) was greater for implanted steers than non-implanted steers (Table 2.2). For the cumulative GrowSafe System period (days 51 to 148), implanted steers had a greater mean ADG compared to non-implanted steers ($P < 0.0001$), and a greater mean ADFI ($P = 0.0150$) (Table 2.2). Low RFI steers had a greater FE than high RFI steers ($P = 0.0159$) and FE was significantly increased with steroid treatment ($P < 0.0001$) (Table 2.2). Low RFI steers had a greater mean body weight than steers of high RFI status at the end of the second implant phase ($P = 0.0391$). Steroid-treated steers also had a greater body weight at the end of the second implant phase than non-treated steers ($P < 0.0001$) (Table 2.2).

Because steers were placed onto RH supplementation by block, steer weights at the beginning of the RH treatment period did not coincide with those at the end of the second implant phase. Steer weight at initiation of RH supplementation was greater for implanted steers than non-

implanted steers ($P < 0.0001$) (Table 2.3). ADG and FE during the four-week RH supplementation period was unaffected by treatment ($P > 0.05$), although ADFI was lower in low RFI steers than in high RFI steers ($P = 0.0241$) (Table 2.3). Steer weight after the RH supplementation was significantly greater in implanted steers than non-implanted steers ($P < 0.0001$) even with weight at initiation of RH supplementation as a covariate (Table 2.3).

Implantation resulted in a higher mean slaughter weight ($P < 0.0001$), despite a significant interaction between RFI and RH treatments ($P = 0.0278$) in which the means of the different combinations of interaction were not significantly different ($P > 0.05$) (Table 2.3). There was a significant interaction between RFI, steroid implant and RH treatment (P = 0.0446) on mean HCW where implanted low RFI steers not treated with RH and implanted high RFI steers treated with RH had the highest weights (383 ± 6.98 and 386 ± 8.12 respectively) (Figure 2.7). Steers that received RH consumed less than the anticipated treatment amount of 200 mg/head/day. The mean intake of RH was 147.12 ± 4.28 mg/head/day and there was no effect of RH on growth and production measurements ($P > 0.05$). RH supplementation did not affect steer body weight (Table 2.3). For the full Growsafe period, which included the 1st and 2nd implant phases and the RH supplementation phase (day 51 to the end of study), mean ADG and ADFI were higher in implanted steers than in non-implanted steers ($P < 0.0001$ and 0.0115, respectively) (Table 2.3). Mean FE was also higher in steroid treated steers than in non-treated steers ($P < 0.0001$). From day 0 to end of study, ADG was higher in implanted steers than in non-implanted steers ($P < 0.0001$) (Table 2.3).

Table 2.3 Least squares means (with standard errors of the means) of growth and carcass characteristics as affected by steer RFI status, steroid and ractopamine hydrochloride treatments during ractopamine supplementation (last 28 days) and for whole study.

Measurement	RFI		Steroid		Ractopamine hydrochloride (RH)		P values[1]			P values[1]
	Low	High	Steroid	Control	RH	Control	RFI	Steroid	RH	RFI × RH
n[2]	26	21	23	24	23	24				
Ractopamine feeding period only										
Body weight before RH treatment, kg	628.00 (3.73)	626.00 (4.15)	657.00 (4.00)[a]	597.00 (3.90)[b]	630 (3.99)	624 (3.90)	0.6018	<0.0001	0.3599	0.1923
Average daily gain, kg	1.40 (0.13)	1.61 (0.13)	1.82 (0.19)	1.19 (0.18)	1.40 (0.12)	1.61 (0.12)	0.2334	0.0724	0.2418	0.1000
Average daily feed intake, kg/head	16.00 (0.33)[a]	17.20 (0.37)[b]	16.30 (0.55)	16.90 (0.53)	16.20 (0.35)	17.00 (0.33)	0.0241	0.5952	0.1243	0.1514
Feed conversion efficiency (gain: feed intake)	0.087 (0.007)	0.093 (0.007)	0.110 (0.011)	0.071 (0.011)	0.087 (0.007)	0.093 (0.007)	0.4831	0.2907	0.4126	0.0508
Body weight after RH treatment, kg	667.00 (4.52)	669.00 (5.20)	704.00 (5.01)[a]	633.00 (4.73)[b]	668.00 (4.84)	668.00 (4.90)	0.7748	<0.0001	0.6815	0.1932
Cumulative day 51 to end of study										
Average daily gain, kg	1.55 (0.04)	1.50 (0.04)	1.69 (0.03)[a]	1.37 (0.03)[b]	1.52 (0.04)	1.53 (0.04)	0.3406	<0.0001	0.8179	0.3469
Average daily feed intake, kg/head	16.40 (0.26)	16.30 (0.27)	16.90 (0.28)[a]	15.90 (0.27)[b]	16.20 (0.28)	16.60 (0.27)	0.7494	0.0115	0.3917	0.3314
Feed conversion efficiency (gain: feed intake)	0.093 (0.002)	0.092 (0.002)	0.100 (0.002)[a]	0.086 (0.002)[b]	0.093 (0.002)	0.093 (0.002)	0.5589	<0.0001	0.9882	0.1110
Cumulative day 0 to end of the study										
Average daily gain, kg	1.49 (0.02)	1.45 (0.03)	1.65 (0.03)[a]	1.29 (0.02)[b]	1.46 (0.03)	1.48 (0.02)	0.4144	<0.0001	0.7535	0.0642
Slaughter weight, kg	669.00 (4.03)	667.00 (4.64)	704.00 (4.43)[a]	632.00 (4.19)[b]	667.00 (4.31)	669.00 (4.34)	0.7692	<0.0001	0.6815	0.0278
Hot carcass weight, kg	359.00 (4.25)	362.00 (4.57)	382.00 (4.41) [a]	339.00 (4.34) [b]	363.00 (4.41)	359.00 (4..34)	0.6480*	<0.0001*	0.3879*	0.0446

[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.

[1] Probability of the F test, with significance at $P \leq 0.05$.

[2]Steer removed from study was of low RFI status, implanted and supplemented with ractopamine hydrochloride.

2.6.2 Meat quality characteristics

Meat quality characteristics of both GM and SM muscles were measured at days 3 and 12 *post-mortem*. In the GM muscle, intramuscular pH, temperature, drip loss, cooking loss, *L**, *a**, *b**, chroma, moisture, protein and fat contents were not affected by RFI status, steroid implantation, or RH supplementation ($P > 0.05$) (Table 2.4). A three-way interaction was observed between steroid, RH and DOA on the purge loss percentage of the GM muscle ($P = 0.0272$), where mean purge loss percentage was greatest with *post-mortem* ageing from steers in all treatments except those that received RH only (Figure 2.8). Ash content was increased in GM muscles from non-implanted steers ($P = 0.0453$). Mean hue value for the GM muscles was higher in low RFI steer carcasses than from high RFI steer carcasses ($P = 0.0281$), but unaffected by steroid or RH application ($P > 0.05$) (Table 2.4). Steroid implants influenced the WBSF values of the GM muscles ($P = 0.0405$), where muscles from implanted steers had a greater mean WBSF value than muscles from non-implanted steers (Table 2.4). Neither RFI status nor supplementation with RH influenced mean WBSF values in the GM muscles ($P > 0.05$). (Table 2.4).

Table 2.4 Least squares means (with standard errors of the means) of meat quality characteristics in the *gluteus medius* (GM) muscle as affected by steer RFI status, steroid and ractopamine hydrochloride treatment.

Measurement	RFI		Steroid		Ractopamine hydrochloride (RH)		*P* values[1]		
	Low	High	Steroid	Control	RH	Control	RFI	Steroid	RH
n[2]	25	21	22	23	22	24			
pH	5.57 (0.03)	5.61 (0.03)	5.61 (0.03)	5.57 (0.03)	5.58 (0.03)	5.590 (0.03)	0.2930	0.2561	0.9510
Temperature (°C)	5.83 (0.23)	6.14 (0.25)	5.92 (0.242)	6.08 (0.237)	5.91 (0.24)	6.09 (0.24)	0.3988	0.6238	0.6066
Purge loss (%)	1.39 (0.17)	1.46 (0.19)	1.44 (0.18)	1.41 (0.18)	1.62 (0.18)	1.24 (0.18)	0.7279*	0.8860	0.0831*
Drip loss (%)	1.25 (0.09)	1.38 (0.11)	1.36 (0.10)	1.28 (0.10)	1.33 (0.11)	1.31 (0.10)	0.3487	0.5867	0.9060
Cook loss (%)	24.52 (0.72)	25.46 (0.81)	25.43 (0.77)	24.55 0.75	24.57 (0.77)	25.42 (0.75)	0.3883	0.4204	0.4338
L*	35.12 (0.35)	34.35 (0.38)	34.75 (0.37)	34.72 (0.37)	34.42 (0.38)	35.03 (0.36)	0.1430	0.9544	0.3361
a*	22.73 (1.11)	24.40 (1.21)	22.58 (1.16)	24.56 (1.15)	22.56 (1.18)	24.57 (1.13)	0.3099	0.2315	0.2248
b*	4.95 (0.25)	4.52 (0.28)	4.79 (0.26)	4.68 (0.26)	4.55 (0.27)	4.92 (0.26)	0.6173	0.7700	0.3373
Chroma	23.11 (0.37)	23.84 (0.41)	23.05 (0.39)	23.40 (0.39)	22.93 (0.40)	24.01 (0.38)	0.1931	0.1298	0.0572
Hue	11.29 (0.43)[a]	11.10 (0.48)[b]	11.32 (0.45)	11.07 (0.45)	10.72 (0.46)	11.68 (0.44)	0.0281	0.7048	0.1378
Protein (%)	22.10 (0.80)	22.00 (0.87)	21.40 (0.84)	22.80 (0.83)	21.90 (0.85)	22.20 (0.82)	0.8964	0.2444	0.8181
Fat (%)	3.52 (0.33)	4.01 (0.36)	3.32 (0.35)	4.21 (0.35)	3.56 (0.36)	3.97 (0.34)	0.3321	0.0796	0.4031
Ash (%)	1.18 (0.05)	1.20 (0.05)	1.12 (0.05[a])	1.27 (0.05[b])	1.19 (0.05)	1.19 (0.05)	0.8437	0.0453	0.9465
Moisture (%)	73.10 (0.82)	72.80 (0.90)	74.20 (0.86)	71.80 (0.86)	73.30 (0.88)	72.60 (0.84)	0.7795	0.0523	0.5702
WBSF (N)[3]	37.40 (1.52)	34.99 (1.59)	37.75 (1.55[a])	34.64 (1.54[b])	35.15 (1.56)	37.25 (1.53)	0.1247	0.0405	0.1651

[*] Main effects involved in interactions have their *P* value marked with an asterisk (*).
[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.
[1] Probability of the F test, with significance at $P \leq 0.05$.
[2]Steer removed from study was of low RFI status, implanted and supplemented with ractopamine hydrochloride. GM muscle removed from study was from a steer of low RFI status, not implanted but supplemented with ractopamine hydrochloride.
[3]WBSF, Warner-Bratzler shear force.

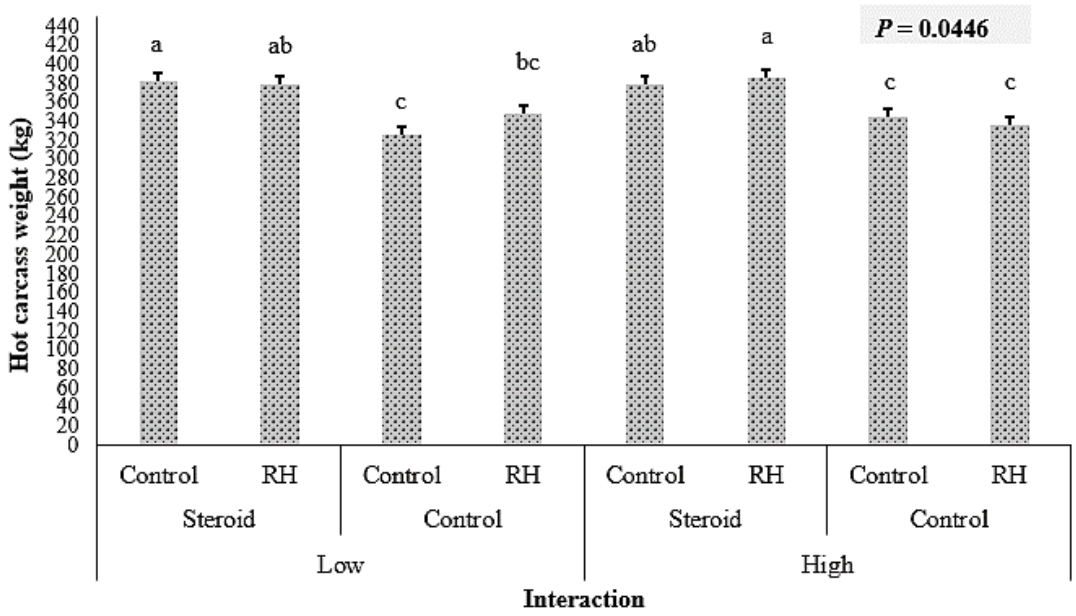

Figure 2.7 Hot carcass weight from steers selected for RFI, and subjected to steroid implantation or not and RH supplementation or not. (Carcasses from steers of Low RFI status, implanted and not RH supplemented (n = 6); Muscles from steers of low RFI status, non-implanted and RH supplemented (n = 7); Muscles from steers of low RFI status, non-implanted and not RH supplemented (n = 6); Muscles from steers of low RFI status, implanted and RH supplemented (n = 7); Muscles from steers of high RFI status, implanted and RH supplemented (n = 5); Muscles from steers of high RFI status, non-implanted and not RH supplemented (n = 6); Muscles from steers of high RFI status, implanted and not RH supplemented (n = 5); Muscles from steers of high RFI status, non-implanted and RH supplemented (n = 5).

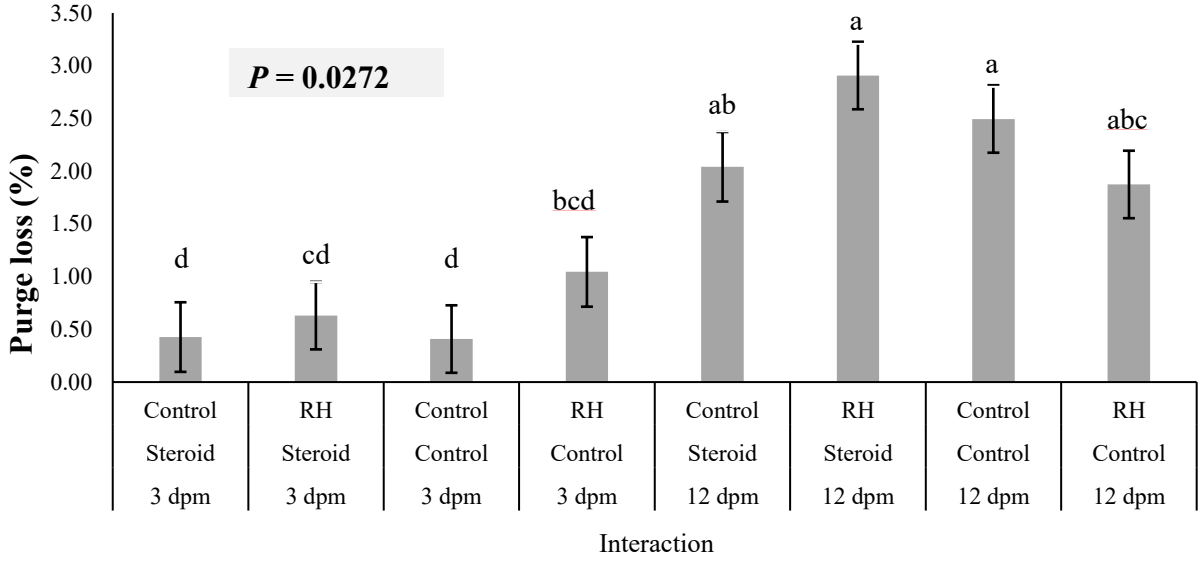

Figure 2.8 Interaction between RFI status, steroid implant and ageing for 3 or 12 days post-mortem (dpm) for mean purge loss (%) from GM muscles (Implanted without RH supplementation and aged for 3 dpm, n = 7; Implanted with RH supplementation and aged for 3 dpm, n= 6; Not implanted with RH supplementation and aged for 3 dpm, n = 6; Implanted without RH supplementation and aged for 12 dpm, n = 5; Implanted with RH supplementation and aged for 12 dpm, n = 5; Not implanted without RH supplementation and aged for 12 dpm, n = 6; Not implanted with RH supplementation and aged for 12 dpm, n = 6). Treatment interaction with different letters differ using the Tukey's method for least square mean comparisons between interaction means ($P < 0.05$).

In the SM muscle, intramuscular pH, purge loss, drip loss and cooking loss, *L**, *a**, *b**, chroma, hue, fat and ash contents were not affected by RFI status, steroid implantation, or RH supplementation, nor by their interactions ($P > 0.05$) (Table 2.5). Two-way interactions were found between RFI and RH supplementation, for muscle temperature recorded at pH measurement ($P = 0.0440$) (Figure 2.9) where mean muscle temperature was higher in the SM muscles of low RFI

steers that received RH treatment than that from low RFI steers that did not receive RH treatment (P = 0.0125) (Table 2.5), for protein content of the SM muscle where it was greatest in high RFI steers supplemented with RH than low RFI steers not supplemented with RH (P = 0.0016) (Figure 2.10). For moisture content however, although a significant interaction resulted between RFI and RH supplementation of the SM muscle (P = 0.0290) (Figure 2.11), means were not significantly different. There was an interaction between steroid implant use and DOA (P = 0.0187), which indicated that the WBSF from steers implanted with steroids was greater than that of control steers at 3 dpm, but the same as that of the control steers at 12 dpm (Figure 2.12). Low RFI steers had significantly different mean WBSF values (P = 0.0060), with WBSF greatest in low RFI steers (P = 0.0060) (Table 2.5).

Table 2.5 Least square means (with standard errors of the means) of meat quality characteristics in the *semimembranosus* (SM) muscle as affected by steer RFI status, steroid and ractopamine hydrochloride treatments.

Measurement	RFI		Steroid		Ractopamine hydrochloride (RH)		*P* values[1]		
	Low	High	Steroid	Control	RH	Control	RFI	Steroid	RH
n[2]	26	21	23	24	23	24			
pH	5.55 (0.02)	5.55 (0.02)	5.56 (0.02)	5.55 (0.02)	5.56 (0.02)	5.55 (0.02)	0.9986	0.9038	0.8660
Temperature (°C)	5.55 (0.27)	5.67 (0.27)	5.66 (0.27)	5.56 (0.27)	5.86 (0.27)[a]	5.36 (0.27)[b]	0.5497	0.6306	0.0125*
Purge loss (%)	1.85 (0.15)	1.75 (0.18)	1.84 (0.17)	1.76 (0.16)	1.86 (0.17)	1.75 (0.16)	0.6777	0.7159	0.6475
Drip loss (%)	1.370 (0.10)	1.372 (0.11)	1.38 (0.11)	1.36 (0.11)	1.29 (0.11)	1.44 (0.11)	0.9916	0.8889	0.3099
Cook loss (%)	28.60 (1.02)	27.32 (1.13)	27.35 (1.09)	28.57 (1.06)	27.35 (1.09)	28.57 (1.06)	0.5365	0.4247	0.5474
L*	34.88 (0.28)	34.18 (0.31)	34.84 (0.30)	34.22 (0.29)	34.57 (0.30)	34.49 (0.29)	0.1039	0.1526	0.8517
a*	23.32 (1.20)	23.24 (0.22)	23.24 (0.21)	23.32 (0.21)	23.19 (0.21)	23.37 (0.20)	0.7840	0.7757	0.7757
b*	5.06 (0.22)	23.81 (0.25)	5.16 (0.24)	4.68 (0.23)	5.06 (0.22)	4.98 (0.25)	0.8045	0.4234	0.8736
Chroma	23.92 (0.22)	23.81 (0.25)	23.82 (0.24)	23.91 (0.23)	23.80 (0.24)	23.93 (0.23)	0.7187	0.7957	0.6992
Hue	11.94 (0.47)	11.91 (0.52)	12.28 (0.50)	11.56 (0.49)	11.94 (0.50)	11.91 (0.49)	0.9561	0.3052	0.9706
Protein (%)	22.30 (0.24)	22.70 (0.27)	22.50 (0.26)	22.50 (0.25)	22.60 (0.26)	22.50 (0.25)	0.2173*	0.9386	0.7249*
Fat (%)	2.58 (0.29)	2.81 (0.32)	2.51 (0.31)	2.89 (0.30)	2.71 (0.31)	2.69 (0.30)	0.5963	0.3932	0.9702
Ash (%)	1.66 (0.21)	1.16 (0.23)	1.47 (0.23)	1.36 (0.22)	1.47 (0.23)	1.36 (0.22)	0.1225	0.7253	0.6350
Moisture (%)	73.50 (0.41)	73.30 (0.45)	73.50 (0.44)	73.2 (0.43)	73.20 (0.44)	73.50 (0.43)	0.7712*	0.6393	0.6331*
WBSF (N)[3]	46.30 (1.41[a])	42.10 (1.47[b])	46.60 (1.45[a])	41.90 (1.49[b])	44.53 (1.42)	43.93 (1.43)	0.0060	0.0014*	0.6811

Main effects involved in interactions have a *P* value marked with an asterisk (*).
[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.
[1] Probability of the F test, with significance at $P \leq 0.05$.
[2]Steer removed from study was of low RFI status, implanted and supplemented with ractopamine hydrochloride. GM muscle removed from study was from a steer of low RFI status, not implanted but supplemented with ractopamine hydrochloride.
[3]WBSF: Warner-Bratzler shear force

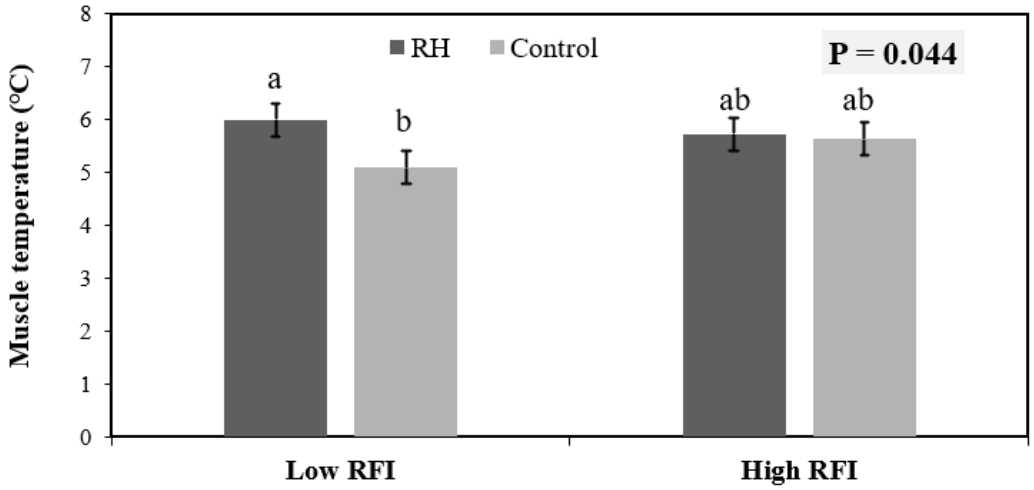

Figure 2.9 Interaction effects between RFI status and RH supplementation on mean muscle temperature at fabrication of SM muscles from cross-bred Angus steers (Low RFI and RH supplemented, n=13; Low RFI and not RH supplemented, n =13; High RFI and RH supplemented, n=10; High RFI and not RH supplemented, n=11).

Treatment means with different superscripts differ according to least squares mean differences ($P < 0.05$)

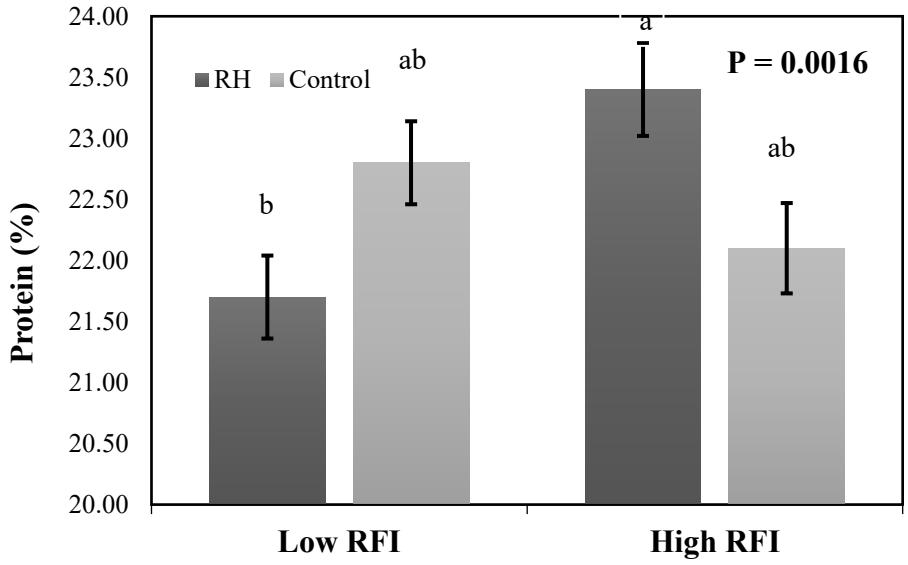

Figure 2.10 Interaction effects between RFI status and RH supplementation on mean muscle protein content from proximate analyses at 3-dpm (Low RFI and RH supplemented, n = 13; Low RFI and not RH supplemented, n = 13; High RFI and RH supplemented, n = 10; High RFI and not RH supplemented, n = 11).

Treatment means with different superscripts differ according to least squares mean differences ($P < 0.05$).

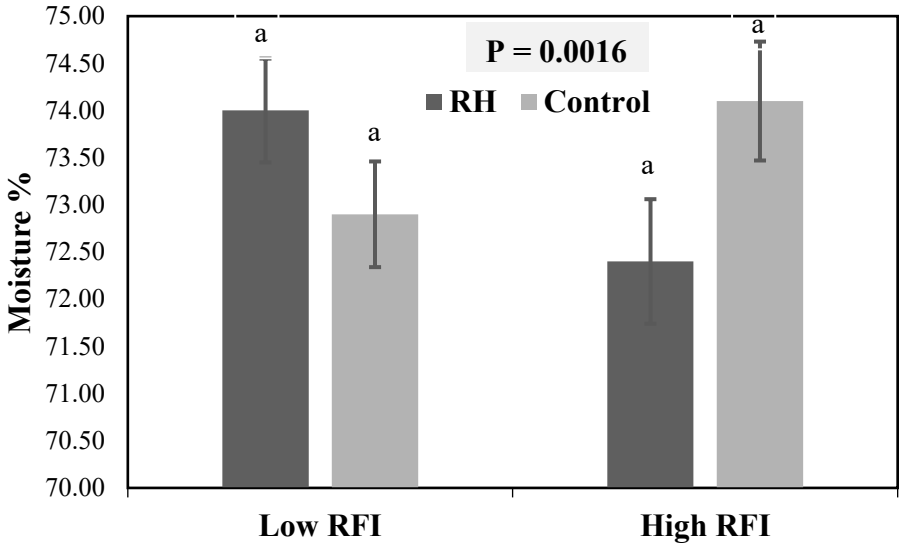

Figure 2.9 Interaction effects between RFI status and RH supplementation on mean muscle moisture content from proximate analyses at 3-dpm (Low RFI and RH supplemented, $n = 13$; Low RFI and not RH supplemented, $n = 13$; High RFI and RH supplemented, $n = 10$; High RFI and not RH supplemented, $n = 11$).

The effect of *post-mortem* ageing on meat quality of GM and SM muscle characteristics is shown in Table 2.6. Regardless of muscle type, muscle pH and cooking loss percentage were unaffected by *post-mortem* ageing (Table 2.6). Muscle temperature at pH measurement and drip loss percentage were highest at day 3 *post-mortem* for both muscles ($P < 0.05$) (Table 2.6), with the decrease in SM WBSF observed in muscles from steroid treated steers only (Figure 2.12), while GM WBSF decreased with DOA without interaction ($P < 0.0001$). Purge loss percentage increased with DOA in the SM, as did mean L^*, b^* and hue values in both muscles ($P < 0.05$) (Table 2.6). Muscle chroma and a* values were unchanged with ageing in the GM ($P > 0.05$) but increased with DOA in the SM ($P < 0.05$) (Table 2.6).

Table 2.6 Least square means (with standard errors of the means) of meat quality characteristics as affected by 3- and 12-days *post-mortem* ageing.

Measurement	*Post-mortem* ageing period		*P* value[1]
	3 days	12 days	
***Gluteus medius* (GM) muscle**			
n[2]	46	46	-
pH	5.62 ± 0.05	5.56 ± 0.05	0.1074
Temperature (°C)	6.30 ± 0.32	5.70 ± 0.31	0.0661
Purge loss (%)	0.61 ± 0.18	2.24 ± 1.18	< 0.0001*
Drip loss, %	1.64 ± 0.10[a]	1.00 ± 0.10[b]	< 0.0001
Cook loss (%)	37.46 ± 2.03	36.84 ± 2.03	0.7609
L*	33.38 ± 0.49[a]	36.11 ± 0.49[b]	< 0.0001
a*	22.60 ± 1.22	24.50 ± 1.22	0.2437
b*	4.02 ± 0.36[a]	5.45 ± 0.26[b]	< 0.0001
Chroma	23.15 ± 0.63	23.73 ± 0.63	0.2395
Hue	9.72 ± 0.86[a]	12.65 ± 0.86[b]	< 0.0001
WBSF (N)[3]	40.06 ± 1.53[a]	32.33 ± 1.54[b]	< 0.0001
Semimembranosus* (SM) *muscle			
n	47	47	-
pH	5.55 ± 0.03	5.55 ± 0.03	0.9916
Temperature (°C)	5.99 ± 0.27[a]	5.23 ± 0.27[b]	0.0001
Purge loss (%)	0.68 ± 0.16[a]	2.92 ± 0.17[b]	< 0.0001
Drip loss, %	1.73 ± 0.11[a]	1.00 ± 0.11[b]	< 0.0001
Cook loss (%)	45.70 ± 20.55[a]	73.05 ± 20.55[b]	0.3323
L*	33.27 ± 0.31[a]	35.80 ± 0.31[b]	< 0.0001
a*	22.75 ± 0.33[a]	23.59 ± 0.33[b]	0.0014
b*	4.31 ± 0.40[a]	5.65 ± 0.40[b]	< 0.0001
Chroma	23.16 ± 0.40[a]	24.31 ± 0.40[b]	< 0.0001
Hue	10.44 ± 0.86[a]	13.32 ± 0.86[b]	< 0.0001
WBSF (N)	47.00 ± 1.41[a]	41.5 ± 1.41[b]	0.0001*

Measurements where ageing period is involved in an interaction are indicated with an asterisk (*) next to their *P* values

[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.

[1] Probability of the F test, with significance at $P \leq 0.05$.

[2]Steer removed from study was of low RFI status, implanted and supplemented with ractopamine hydrochloride. GM muscle removed from study was from a steer of low RFI status, not implanted but supplemented with ractopamine hydrochloride.

[3]WBSF: Warner-Bratzler shear force.

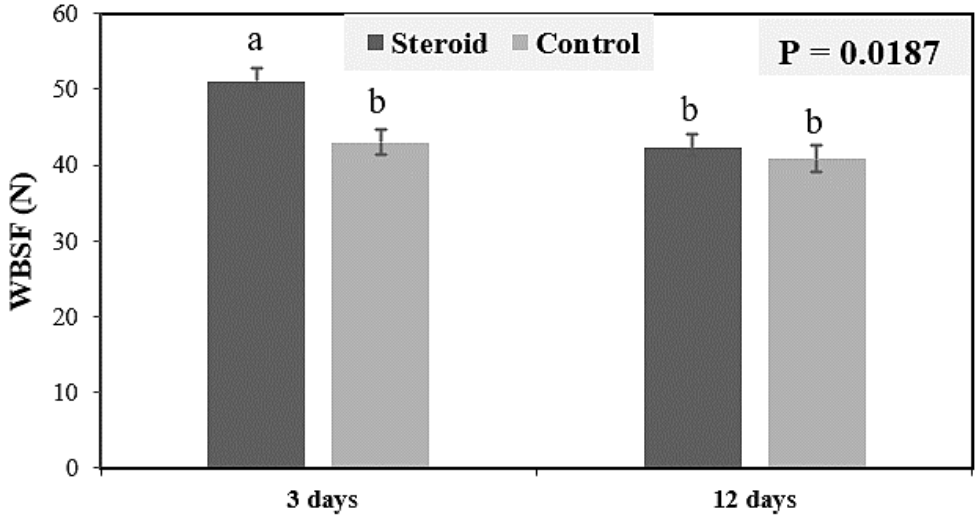

Figure 2.12 Interaction between steroid implant and post-mortem ageing period on mean Warner-Bratzler Shear Force (WBSF) of SM muscles.

Treatment interaction with different letters differ using the Tukey's method for least square mean comparisons between interaction means ($P < 0.05$).

2.6.3 Pearson's correlations

Correlations between growth and meat quality measurements were considered significant at $P \leq 0.001$. Of significance include strong positive correlations between RFI and feed intake during the first (r = 0.77) and second (r = 0.65) implant phases, the combined implant phases (r = 0.73), the RH supplementation phase (r = 0.49) and the entire study period (r = 0.60). Moderate positive correlations were also found between feed intake and body weight at the end of the first implant phase (r = 0.55), at the end of the second implant phase (r = 0.55), and after RH supplementation (r = 0.59). There were also strong positive correlations found between feed intake and average daily weight gain during the entire implant period (r = 0.61), for the entire study (including RH supplementation) (r = 0.66) and slaughter weight (r = 0.60) ($P < 0.0001$). Moderate and strong positive correlations were also found between FE and final weight at the end of the second implant phase (r = 0.57), final weight after RH supplementation (r = 0.69), average daily weight gain during the entire implant phase (r = 0.68), average daily gain during the entire study

(implant and RH supplementation phases) (r = 0.82), slaughter weight (r = 0.69) and carcass weight (r = 0.50) all at P <0.0001.

For GM meat quality characteristics, a moderate positive correlation was found between feed intake during the entire study (implant and RH supplementation phases) and the cook loss percentage at day 3 *post-mortem* (r = 0.55) ($P < 0.0001$). Negative moderate correlations existed between L^* of the GM muscle at 12 dpm and average daily gain during RH supplementation (r = -0.48) ($P = 0.0007$) and FCE during RH supplementation (FER) (r = -0.53) ($P = 0.0007$). In the SM muscle at 3 dpm, initial body weight at first steroid application was moderately positively correlated with muscle temperature at pH measurement (r = 0.48) ($P = 0.0007$). Average daily weight gain during the entire study (implant and RH supplementation phases) was moderately positively correlated with WBSF (r = 0.47) ($P = 0.0007$).

2.6.4 Average monthly temperatures

The study began on April 4, 2016, with implantation of treated steers. The second implantation occurred 90 days later on July 4, 2016. Administration of RH began with the first block on August 31 (study day 149) and ended for the last block November 2, 2016. Minimum, maximum and average monthly temperatures obtained for the Kinsella Research Station from the Alberta Climate Information Service, an Alberta Government weather station electronic archive (https://acis.alberta.ca/weather-data-viewer.jsp) are shown in Figure 2.12. The highest average daily temperatures occurred in June, July and August in 2016.

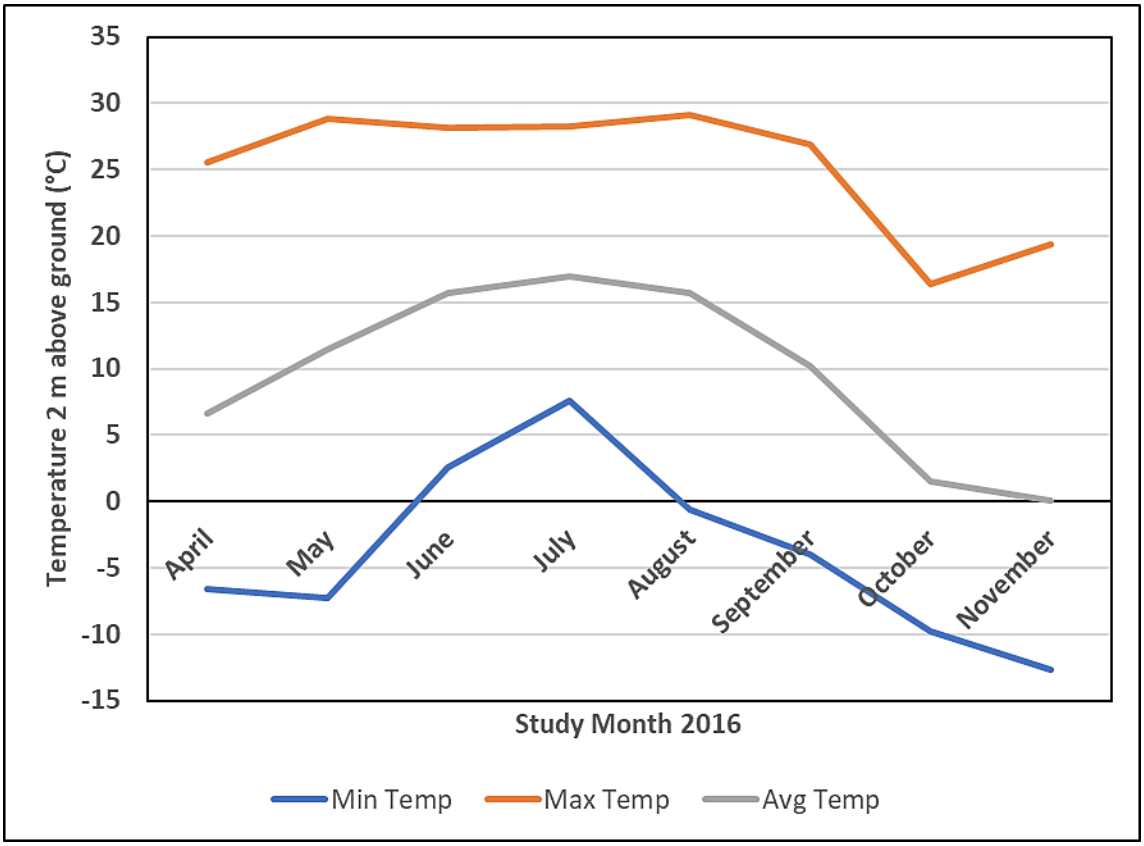

Figure 2.10 Minimum, maximum and average daily temperature for study live animal phase in 2016.

2.7 Discussion

In animal production, feed represents the largest input expense as it constitutes 50-70% of total production cost (Cattle Fax, 2017). Steroids and β-adrenergic agonists (β-AA) are the two most common growth promotants used in the North American beef industry to increase feed efficiency (FE), and are often used within the same production system. In a survey by Samuelson et al. (2016) involving 24 feedlot nutritionists who provided services to more than 14 million fed cattle annually across the United States, 84.8% of the feedlots surveyed supplemented their finishing steers with a β-AA. Out of this number, 95.5% used RH where it was commonly fed for a duration of 28 days, just as it was in the current study. Much research has substantiated their effectiveness (Bass *et al.,* 2009; Lean *et al.,* 2014; Hagenmaier *et al.,* 2017). Furthermore, selecting for low RFI has offered an additional strategy for increasing FE without indirectly selecting for increased mature animal size (Arthur and Herd, 2008).

In the present study, differences due to selection for low RFI were most evident during the second implantation period, when low RFI steers showed a greater FE than high RFI steers that led to a cumulative increase in FE by the end of the second implant period. Pearson correlation coefficient analysis showed moderate to high positive correlations between RFI and feed intake in the individual steers during the combined growth promotion periods that ranged between 0.49 and 0.77. Our result is corroborated by Hoque et al. (2005) and Nkrumah et al. (2007), where diverse cattle breeds had positive correlations between RFI and DMI that ranged from 0.62 to 0.72. This result is further corroborated by a study by Nkrumah et al. (2004) where high RFI steers consumed about 15% more feed than steers of low RFI status when fed a concentrate-based diet. Despite the significant correlations between RFI and feed intake, low RFI steers in the current study did not consume less feed as anticipated throughout the entire study period, although they consumed less feed during the RH supplementation phase. Low RFI steers had a greater mean FE, however, that was evident at the end of the second implant period and cumulatively for the study. This result is corroborated by the results of Nkrumah et al. (2004), where steers of low RFI status had a feed conversion ratio that was 17% less than steers of high RFI status. Expression of RFI has been shown to be repeatable across the various stages of maturity in both beef steers and heifers (Cassady *et al*., 2016) and male dairy calves (Asher *et al*., 2018), although RFI value has been

shown to change in individual beef cattle between serial feeding periods (Durunna *et al.,* 2012). Durunna et al. (2011, 2012) concluded that evaluation of RFI in steers and heifers would be best performed late post weaning, and the present study agrees with this conclusion given that the greatest expression of RFI divergence was observed during the second steroid and RH supplementation periods.

Interactions between selection for low RFI and steroid application were found in the second steroid period for average daily gain and average daily feed intake, where low RFI steers exhibited a greater mean average daily gain and FE than high RFI steers when implanted with sex steroids. These results suggest that average daily feed intake in low RFI steers may be more responsive to an androgenic implant than in high RFI steers. Steroids are known to increase DMI of implanted cattle, leading to an increase in average daily weight gain, body weight gain and ultimately carcass weight (Wileman *et al*., 2009), but this is the first study of which the authors know that has identified increased efficacy of steroids in low RFI steers. Low RFI steers are notably such because they consume less feed than anticipated for their body size and growth rate (Herd and Arthur, 2009); therefore, these cattle may offer the opportunity for the largest gains in ADFI with steroid application. The interactions between RFI and steroid application support this hypothesis, as they indicated that the effects of selection for low RFI and the application of steroids were cumulative for ADFI and ADG. There was not a concomitant cumulative effect on FE, however, although selection for low RFI and steroid application still increased FE during the second implant period respectively as main effects.

Although RFI increased FCE, steer growth was most affected by the use of steroids. According to Nichols et al. (2002), estrogenic and androgenic hormones have been employed in the production of beef for over 50 years to improve efficiency. Steroid implants provide producers an opportunity to increase not only the biological, but also the economic efficiency of beef cattle (Preston, 1987). As expected, steroids increased ADG, ADFI and body weight, their effect exceeding that of selection for low RFI. Interestingly, during the first steroid phase, gains in body weight made with the estrogenic steroid were not accompanied by an increase in FCE, while those observed with the androgenic second steroid were. This was unexpected, as steroids have been

found to increase FE (Nichols *et al.,* 2002). Estrogenic steroids have been found to be beneficial in the stimulation of increased gain by enhancing FCE in beef cattle (Ferguson *et al.,* 1988). Apple et al. (1991) and Bass et al. (2009) compared both estrogenic (20 mg estradiol and 200 mg progesterone) and androgenic (24 mg estrogen and 120 mg trenbolone acetate) implants in their respective studies and found no difference in final body weight, cumulative weight gain, and ADG of Holstein steers. The first implant phase in the current study was during the spring (April to June), which had lower average monthly temperatures than the second implant period (June to August). About 60 – 70% of heat from the body's metabolic activities occur in the heart, liver, kidneys and brain (Sjaastad *et al.,* 2003), with demands increasing as ambient temperatures decrease. Heat is additionally produced in the gut of ruminants during fermentation by bacteria, especially in the rumen (Czerkawski, 1980). Reduced ambient temperatures during the first implant period may have routed energy away from growth toward maintenance, thereby limiting any increase in the FE of implanted steers.

Sex steroid hormones have been shown in numerous studies to increase levels of circulating IGF-1 (Pell, 1989; McLaughlin *et al.,* 1991, 1993; Pampusch *et al.,* 2003). According to Hayden et al. (1992), Hongerholt et al. (1992) and Pampusch et al. (2003), hormonal implants such as trenbolone acetate with estrogen exert their effect through IGF-1. Detection of IGF-1 concentration in biological tissue can be complicated by it being bound to an IGFBP, which impedes its detection by immunoassay (Hossner *et al.,* 1997). Circulating IGF-1 is bound to IGFBP-3, which is one of six identified insulin-like growth factor binding proteins, and increases IGF-1 half-life in circulation (Baxter, 1991; Hossner *et al.,* 1997). A known mitogen for cell proliferation, IGF-1 has been found to be both genetically and phenotypically positively correlated with RFI in heifers and growing bulls (Brown *et al.,* 2004; Moore *et al*., 2005). Our results from the quantification of circulating IGF-1 do not agree with studies by Johnson et al. (1996a, 1996b) as IGF-1 and IGFBP-3 concentrations in the current study were similar for implanted and non-implanted steers. Our results arose from a single blood sampling, and although a single sampling is sufficient to determine circulating IGF-1 concentration, it is recommended to collect several samples over a period of 6 to 8 h to accurately quantify IGF-1 levels (Hossner *et al*., 1997). Johnson et al. (1996a) reported that implantation of crossbred beef steers with 120 mg of TBA and 24 mg of E_2 increased

circulating trenbolone acetate within 2 days of implantation, with its concentration subsiding by almost half by day 21 post-implantation. Circulating IGF-1 levels were measured after the 2nd implant and circulating estrogen levels tend to persist with combination implants (Johnson *et al*., 1996a; Smith *et al*., 2019; Smith and Johnson, 2020). In humans, estradiol is known to lower circulating IGF-1, while testosterone increases it (Veldhuis et al., 2005). Persistent estrogen from the first implant may have exerted a dampening additive effect on IGF-1 with estrogen in the 2nd implant, although the anticipated rapid increase in TBA makes this unlikely (Johnson *et al*., 1996b). Understanding the effects of serial implants on IGF-1 warrants investigation as it may lead to more effective use of steroids.

In the current study, RH supplementation did not increase steer growth performance measurements but interacted with steroids and RFI to increase HCW Bass et al. (2006) reported that cattle fed RH at 200 mg/head/day achieved a greater mean live weight than cattle that did not receive RH. In the study by Hagenmaier et al. (2017) supplementation of crossbred *Bos taurus* steers and heifers with RH enhanced ADG and FE by 21.2% and 20% respectively. RH also increased hot carcass weight by 7 kg and *longissimus* muscle area by 4 cm^2. RH in the study by Hagenmaier et al. (2017) was fed at a rate of 400 mg/head/day, and far exceeded the target dose of 200 mg/head/day from the current study, where we observed that neither ADG nor FE was increased by RH supplementation. This may have been due to the steers supplemented with RH receiving less than the recommended amount of 200 mg/head/day over the 28-day supplementation period. Our results also do not completely align with a meta-analysis by Lean et al. (2014) where in more than 50 comparisons, RH enhanced growth performance and resulted in greater hot carcass weight and *longissimus* muscle area consistently. RH in the current study interacted with steroid implant and high RFI to increase HCW. Comparing this interaction and that between implanted low RFI steers not treated with RH, steroid implant appears to be the driving force behind this increase. Despite the results of Lean et al. (2014) and other studies (Bass *et al.,* 2009; Hagenmaier *et al*., 2017), Bittner et al. (2016) reported little effect of RH administration on beef crossbred steers. In two of three separate experiments, Bittner et al. (2016) found no difference in steer final live weight when RH was administered at either 0, 100 or 200 mg/head/day, although FE (gain to feed ratio) increased linearly with RH dose in each experiment. Bittner *et al.* (2016) also reported

that for all three experiments, ADG was not different between the doses, meaning that RH did not increase ADG. The efficacy of RH on steer growth may also have been compromised by the onset of cold temperatures in the present study. The efficacy of RH has been shown to be compromised by heat stress in lambs (Barnes *et al.,* 2019) and may therefore be compromised by cold stress. The incidence of dark cutting, which indicates reduced muscle glycogen content and therefore lowered capacity for growth, is highest in spring and fall due to cattle needing to use additional energy to adjust to increase variation in daily temperatures (Boykin *et al.,* 2017). Based on the results obtained on RH supplementation in this study and of others, further investigation of the effect of RH dose on the growth and feed intake of beef cattle and how its efficacy is affected by ambient temperature is warranted.

With the exception of WBSF, the use of growth promotants had limited effects on the meat quality characteristics of the *gluteus medius* (GM) and *semimembranosus* (SM) muscles in the current study. In both the GM and SM muscles, WBSF was increased in steers that were implanted with steroids, with this difference disappearing with 12 days *post-mortem* ageing in the SM muscle. This is an indication that increase in the toughness of meat as a result of steroid implant utilization can subside with *post-mortem* ageing. In the review by Morgan (1997), mean WBSF value for steaks from implanted cattle were reported to be 0.49 kg higher than that of steaks from non-implanted cattle. Roeber et al. (2000) and Platter et al. (2003) both reported that steaks from steers treated with steroids had significantly higher WBSF values than steaks from non-treated steers. Similarly, Ebarb et al. (2017) reported that *longissimus lumborum* steaks from implanted feedlot heifers were less tender than that from non-implanted heifers, and that the use of growth promotants negatively influenced the acceptance of beef products by consumers. There is an indication from other literature that implanted steaks need about 7 days of *post-mortem* ageing to attain a similar level of tenderness as non-implanted steers (Schneider *et al.,* 2007; Quinn *et al.,* 2008). The results of the current study concur and emphasize the importance of *post-mortem* ageing on the tenderness of beef produced in a conventional North American system.

Why steroid application increased toughness of both muscles was not further investigated in this study, but Ebarb et al. (2017) attributed the reduction in tenderness associated with the use

of steroids in beef cattle to an increase in cross-sectional area of muscle fibres. Calpastatin activity, however, has been reported to be increased in muscles from implanted cattle (Gerken *et al.,* 1995). To achieve growth in muscle, accretion of protein must exceed the breakdown of protein (Boehm *et al.,* 1998). The calpain family is the enzyme system that contributes to the control of protein accretion and degradation (Boehm *et al.*, 1998). According to Boehm et al. (1998), testosterone increases the activity of calpastatin, which is an inhibitor of calpains, resulting in an increase in protein accretion. Increased tenderness has been associated with decreased calpastatin activity *post-mortem* in muscle (Boehm *et al.,* 1998).

If selection of low RFI animals to reduce feed costs and increase production efficiency compromises meat quality, it is important to address such outcomes due to the effect it could have on the economic advantage of low RFI selection (McDonagh *et al.,* 2001). Residual feed intake (RFI) had limited effect on meat quality characteristics in this study and similar results were reported by Jiu et al. (2020), where RFI had little effect on growth, carcass and meat quality characteristics. In the current study, although the WBSF of the GM was unaffected by RFI, the WBSF of the SM from low RFI steers was greater than that of high RFI steers. Richardson et al. (1998) showed with feedlot steers of low and high RFI status that a decrease in subcutaneous fat was associated with selection to reduce RFI. Results from that study however showed that although no difference was observed in the intramuscular fat content and shear force values ($P > 0.05$) between low and high RFI steers, the myofibrillar fragmentation index was lower in muscles from low RFI steers than those from high RFI steers at 1 and 14 dpm due to high calpastatin levels in low RFI steers. The difference in WBSF observed in the SM in the present study was not due to the increased intramuscular fat in the high RFI steers, however, as there was no difference in intramuscular fat due to RFI. McDonagh et al. (2001) proposed a difference in the calpain system between low and high RFI steers to be responsible for the differences in tenderness. Although there was no difference in the activity of m-calpain and μ-calpain in the *longissimus dorsi* (LD) muscle immediately after slaughter in that study, mean calpastatin level in the LD from low RFI steers was higher than that from high RFI steers by 13%. Calpain and calpastatin levels were not measured in the current study, but this is the most likely mechanism for the increased toughness of beef from the low RFI steers. Results of McDonagh et al. (2001) support further investigation

into how calpain and calpastatin levels differ between low and high RFI steer muscles early *post-mortem*.

Between the two muscles studied, only the SM WBSF was increased in steers selected for low RFI, suggesting that the response of WBSF to selection for RFI may vary by muscle and be most expressed in muscles with high IMCT content. While some studies have reported that the eating quality of meat is not correlated with collagen content (Herring *et al.,* 1967; Hunsley *et al.,* 1971), others have reported that muscles high in collagen are tougher than muscles low in collagen (Dransfield, 1977; Light et al., 1985; Nishiumi *et al.,* 1995). Collagen solubility has been reported to have a relatively low (Renand *et al*., 2001; Berge *et al.,* 2007; Chambaz *et al.,* 2003) or insignificant correlation (Young et al., 1994) with cooked meat tenderness. A high positive correlation was however reported by Torrescano et al. (2003) between heat-insoluble collagen and WBSF of raw beef, where heat-solubility of collagen is dependent on collagen crosslinks (Tanzer, 1973). To date, the effects of RFI on the intramuscular collagen content, heat solubility and mature collagen crosslink concentrations have had limited study and, given the WBSF results of the current study, these warrant characterization. Both low and high IMCT muscles should be considered in future research as differences in collagen and its crosslinking may account for the differential effect of RFI effect on beef muscles.

Supplementation with RH also had limited effects on beef quality characteristics in this study and neither the WBSF values for GM nor SM muscles were influenced by it. While our result agrees with those of Quinn et al. (2008) where no difference in WBSF values of steaks from treated and untreated heifers was reported, it is in contrast with the results of Gruber et al. (2008) where increased values of WBSF were reported for steers fed RH. Although Strydom et al. (2009) observed that the treatment with RH increased calpastatin activity, they also noted no difference in mean WBSF values between the *longissimus lumborum* and *semitendinosus* muscles from treated and control animals. The low dose of RH actually received by the steers in the present study is most likely the reason a limited growth response to RH was observed, which then resulted in no observed effect on meat quality.

Days of *post-mortem* ageing had the greatest effect on the meat quality characteristics measured on both GM and SM muscles in the current study and were typical of that expected. Purge loss, *L*, b**, and hue increasing and drip loss and WBSF decreasing with days of ageing, while chroma and a* value increased in the SM only with ageing. These results agree with those of other studies (Mitchell *et al.,* 1991; Oliete *et al.,* 2005; Ebarb *et al.,* 2017). There was an interaction for purge loss between ageing period, steroid application and RH supplementation, with GM muscles from steers that received RH and steroids having the greatest increase in purge loss with 12 dpm ageing. Why this occurred was unclear, as proximate analysis, with the exception of ash content, indicated no difference in muscle composition regardless of treatment. The increase in tenderness with *post-mortem* ageing is largely associated with *post-mortem* proteolysis, particularly that effected by calpain enzymes (Koohmaraie, 1992).

2.8 Conclusions

This study confirmed that use of steroids in beef cattle enhances the production efficiency of crossbred Angus steers. The use of steroids was most effective in steers of low RFI status, as the increase in feed intake by low RFI steers due to implantation translated into increased gain. Notwithstanding that RFI increased feed conversion efficiency, feed conversion efficiency was most increased by the use of steroids. While the use of ractopamine hydrochloride had limited effect on meat quality, the increase in WBSF in response to selection for RFI varied by muscle and was most obvious in the SM, a high intramuscular connective tissue muscle. Resolution of the toughening effect of steroid administration *post-mortem* was also only observed in the SM, suggesting that steroid related toughening can persist in some muscles despite *post-mortem* ageing.

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Chapter 3. Effect of Residual Feed Intake (RFI) and Hormonal Growth Promotants on collagen characteristics and their role in toughness of the *semimembranosus* and *gluteus medius* muscles

3.1 Introduction

The two commonest growth promotants (GPs) used by producers are hormonal implants and β-adrenergic agonists (β-AAs) (Lean *et al*., 2018). Hormonal implants are used routinely to increase average daily gain (ADG), final animal weight and the size of the *longissimus* muscle area (LMA) (Nichols *et al.,* 2002). Beta adrenergic agonists (β-AAs) are also used to increase protein accretion, growth rate and feed efficiency (Mersmann, 1998), with ractopamine hydrochloride (RH) being one of two β-AAs approved for use in growing cattle in North America. Ractopamine hydrochloride is a repartitioning agent that has been studied for over four decades in various livestock species (Anderson *et al.*, 2010). As a repartitioning agent, ractopamine has the capacity to deflect nutrients away from deposition as fat toward muscle protein synthesis (Ricks *et al.,* 1984). There are however some concerns on how the use of hormonal growth promotants affects the quality of beef, including how they may increase toughness as measured by Warner-Bratzler Shear Force (WBSF) and the perception of connective tissue by consumer and trained panels (Watson, 2008). Earlier research has shown that GPs decrease tenderness by increasing the activity of calpastatin (Gerken *et al*., 1995; Strydom *et al.,* 2009). According to Lean et al. (2014), RH increased WBSF by an estimated 0.2 kg. All these factors influence meat tenderness and can affect the general acceptability of beef products by consumers (Lusk *et al.*, 2001).

Another strategy to increase beef cattle feed efficiency has been to select cattle for residual feed intake. Measurement of residual feed intake (RFI), which is defined as the difference between the actual and predicted feed intake of an animal was proposed by Koch et al. (1963) as a measure that allowed for selection of efficiency without increasing animal mature size. According to Baker et al. (2006), some studies have demonstrated that an association exists between RFI and maintenance requirements, gain composition, which reflects the composition of the carcass, and meat tenderness. A study by McDonagh et al. (2001) comparing the meat quality of Angus steers selected for low and high RFI status reported no significant difference in shear force after 0 and 14 days of ageing. Myofibrillar fragmentation indices (MFI), which indicate myofibrillar

aggregation, were however lower in the *longissimus* muscle from low RFI Angus steers, and calpastatin concentration 13% higher. These results suggest that selection for low RFI may influence meat tenderness negatively although Jiu et al. (2020) showed that RFI had limited effect on growth, carcass and meat quality characteristics. Given that results have been equivocal, further studies are needed to ascertain how RFI affects meat tenderness.

Conditioning or ageing of meat has the potential of improving meat tenderness as it allows the activity of proteolytic enzymes, specifically the calpain system, which appears to be the essential proteolytic system responsible for the tenderization of meat *post-mortem* (Geesink *et al.,* 1995). Most studies conducted in recent years have focused mainly on *post-mortem* proteolysis of the cytoskeletal and myofibrillar proteins in muscle fibres. *Post-mortem* proteolysis can be altered to a large extent by *post-mortem* management of meat which include the temperature, and length of time of ageing, making the study of the contribution of IMCT to meat toughness essential. The contribution of IMCT to the texture of meat, although important, was presumed to be invariable in comparison to muscle fibres during the *post-mortem* ageing period of meat (Marsh, 1977b; Bailey, 1989; Purslow, 2005). This is because the nature of the IMCT has previously been considered to have undergone limited changes *post-mortem*, thus forming what is referred to as the background toughness of meat (Ouali *et al*., 1992; Sentanfreu *et al.,* 2002).

Pyridinoline (PYR) and Ehrlich's chromogen (EC) are trivalent crosslinks that provide linkage between two telopeptides and the helix of one more collagen molecule (Eyre, 1987). According to McCormick (1999), mature crosslinks and collagen concentration possess an additive effect on meat toughness. The contribution of the IMCT to beef tenderness arises from the amount of collagen and its heat solubility (Hill, 1996; Bailey and Shimokomaki, 1972; Cross *et al.,* 1973). Collagen crosslink concentrations, however, have been shown to change with chronological age, with these changes linked to reduced heat-solubility of collagen, which results in less tender meat (Cranwell *et al.,* 1996; Nishimura *et al.,* 1996). This is most evident in muscles rich in collagen (Shorthose and Harris, 1990). According to Tanzer (1973), the changes with age in collagen crosslinks are related to the covalent intermolecular bonds. Most of the crosslinks in the collagen portion of connective tissues of young animals are of the Schiff base form and are susceptible to acid and heat (Bailey and Shimokomaki, 1971). These reducible crosslinks are

displaced by or converted into crosslinks that are not reducible by heat as the animal ages, resulting in reduced collagen solubility and tougher beef (Dikeman *et al.,* 1971; Robins *et al.*, 1971).

In the study by Ebarb et al. (2017), collagen characteristics, including mature or heat-stable trivalent crosslinks were not influenced by growth promoting technologies (hormonal implants and RH). Strydom et al. (2009) reported similar results where total collagen and collagen solubility of muscle from steers either supplemented with RH or not were not significantly different. In a study by Roy et al. (2015), however, supplementation with RH was found to decrease the amount of the trivalent cross-link pyridinoline, while the use of hormonal implants had the tendency to increase the amount of pyridinoline in the *gluteus medius* muscle. The densities of another trivalent cross-link, Ehrlich's chromogen in the raw *gluteus medius* muscle and collagen were increased as a result of implantation. In the same study, the use of growth promotants did not influence the density of PYR in the collagen of the *semitendinosus* muscle. This indicates that different muscles respond differently to growth promotants with respect to collagen characteristics.

Several studies have shown that there is either a relatively weak correlation between cooked meat tenderness and collagen heat-solubility (Berge *et al*., 2003; Chambaz *et al,* 2003), or a correlation that is not significant (Young *et al.*, 1994). According to Bosselmann and Möller (1994) and Kirchgessner and Schwarz, (1995), the decrease in the heat solubility of collagen, hence the increase in meat toughness, are related with increased concentration of the cross-link pyridinoline. It is therefore crucial to understand how different crosslinks lead to collagen stability, both in quantity and quality, especially the proportion of crosslinks that change overtime from reducible to mature crosslinks (Westin *et al.,* 2002).

3.2 Objective

This study was conducted to elucidate how collagen and collagen cross-link characteristics are modified, if at all, in response to selection for RFI and application of growth promotant treatments. This study tested the following hypotheses:

1. That collagen concentration and collagen cross-link density increase in response to growth promotant use, which result in tougher beef indicated by increased Warner-Bratzler shear force; and,

2. That *post-mortem* ageing of beef weakens the structure of the connective tissue as indicated by increased intramuscular soluble collagen content which is associated with beef of lower Warner-Bratzler shear force.

3.3 Materials and methods

3.3.1 Experimental design and sample handling

The experimental design, animals and treatments used in this study have been described in detail in the materials and methods section of Chapter 2 of this thesis. From these samples, intramuscular connective tissue was isolated and collagen content, solubility, and PYR and EC crosslinks quantified, and related to meat quality results collected and presented in Chapter 2 as well.

3.3.2 Isolation of intramuscular connective tissue (IMCT)

From the *m. gluteus medius* and *m. semimembranosus,* one steak each of 2.5 cm thickness from meat aged for 3 and 12 days were stored (frozen) at -20 °C for the isolation of the perimysium (and endomysium) component of the intramuscular connective tissue, were thawed overnight. The epimysium was carefully trimmed from each steak and discarded, and the remaining steak was cut into 1 cm^3 cubes. The cubed muscle was blended in 5 volumes (w/v) of deionized water using a laboratory blender (Waring Inc, Fisher Scientific). The homogenate was filtered through a 1 mm^2 metal sieve. The material retained on the metal sieve was considered IMCT, which was blended a second time in deionized water and filtered as before. This step was repeated a third time to obtain a clean (whitish appearance) IMCT. Visible blood vessels were removed from the retained IMCT, which was then blotted dry with Whatman No, 4 filter paper (Fisher Scientific, Fisher Scientific, Mississauga, Ontario). The wet weight of the IMCT was obtained, after which it was stored at -20 °C.

3.3.3 Heat solubility of collagen

Steaks measuring about 2.5 cm in thickness were removed from each muscle at its fabrication and used for colour measurement. The steaks were then trimmed of epimysium and fat, weighed and cut into 1 cm^3 cubes. The cubes were spread evenly in an aluminium pan and lyophilized for 100 hours. After the samples were lyophilized, they were weighed and ground into fine powder using a Waring laboratory blender (Model 7011C, Waring Commercial, Torrington,

CT) with two to three pellets of dry ice. The ground samples were stored at -20 ℃ until used for collagen solubility.

The total collagen content of a sample was quantified by determination of the hydroxyproline content with a modified version of Hill (1966). Approximately 1.000 ± 0.0030 g (mean with standard deviation) of lyophilized muscle from each muscle in duplicate were put into glass tubes and 20 mL of Ringer's solution was added. Samples were placed in a rack into a 77 ℃ water bath and heated for 63 minutes once an internal sample temperature of 77 ℃ was reached. Samples were vortexed gently every 10 minutes and, after heating, samples were cooled for 15 minutes in an ice water slurry. Dry and empty centrifuge tubes were weighed and recorded, and the collagen content transferred into them. The centrifuge tubes with samples were then centrifuged for 10 minutes at 3500 g at 4 ℃. After centrifugation, the supernatants (soluble collagen fraction) were collected and stored in labelled plastic vials at -20 ℃. The tubes containing the residue were inverted at an angle of 45 ° to drain the Ringer's solution completely. The centrifuge tubes with the residue (insoluble collagen fraction) were weighed and the weight recorded. The residues were collected and stored at -20 ℃.

3.3.4 Acid hydrolysis

Soluble and insoluble collagen samples were thawed overnight at approximately 4 °C. Approximately 0.3000 ± 0.005 g residue in duplicate were weighed in a labelled tube for hydrolysis. Five (5) mL of 6 M hydrochloric acid (HCl) was added to each tube. For soluble collagen, 1 mL of samples were transferred into labelled tubes. One (1) mL of 12 M HCl was first added, which was followed by 4 mL of 6 M HCl. The content of each tube was vortexed gently, flooded with nitrogen and sealed. The tubes were placed in a dry bath at 110 ℃ and hydrolyzed for 20 hours. After hydrolysis, the tubes were cooled in iced water for 15 minutes. The cooled lysates were filtered using distilled water with Whatman filter paper #4 (Fisher Scientific, Ottawa, ON). The acid solution from each sample was evaporated to dryness using a rotary evaporator at 40 °C and reconstituted in 2 mL distilled water. Minute volumes of 1 M HCl and 2.5 M sodium hydroxide (NaOH) was added to each sample until neutralized, and a pH of 7 was attained with the aid of a litmus paper. The solvent was evaporated from the samples and reconstituted again with 5 mL distilled water. The reconstituted samples were transferred into labelled vials and stored at -20 ℃.

3.3.5 Hydroxyproline quantification

Hydrolyzed soluble and insoluble collagen samples were thawed prior to hydroxyproline quantification according to the method of Neuman and Logan (1950). One (1) mL each of soluble and insoluble collagen hydrolysate in duplicate was used. Hydroxyproline assay standards were prepared by diluting 1 mL of hydroxyproline standard stock solution (400 µg/mL) with 9 mL of deionized water to produce 10 mL of 40 µg/mL of hydroxyproline assay standard. Five (5) mL of this solution were further diluted with 5 mL of de-ionized water to produce 10 mL of 20 µg/mL hydroxyproline assay standard. This was repeated three times in series to produce standards of 10, 5 and 2.5 µg/mL respectively. To obtain the standard curve for the quantification of hydroxyproline, two 1 mL aliquots of each standard were dispensed into test tubes, with 1 mL of deionized water used as the blank. To each tube, 1 mL of 0.01M copper (II) sulfate solution, 1 mL of 2.5 M sodium hydroxide solution, and 1 mL of 6% hydrogen peroxide solution were added to each tube. The tubes were transferred to a water bath at 80 °C for 5 minutes, with frequent vigorous shaking. The tubes were then removed from the water bath and cooled in an ice/water slurry. To each tube, 4 mL of 3.0 N sulfuric acid were added and followed by 2 mL of 5% p-dimethylaminobenzaldehyde for the development of colour. The tubes were then transferred to a 70 °C water bath for 16 minutes, after which they were cooled in cold water for 5 minutes. The absorbances of the final experimental samples were measured at 550 nm immediately and recorded. The hydrolyzed soluble and insoluble collagen samples were subjected to the same procedure and their absorbances recorded. Linear regression equations derived from absorbances of samples with known hydroxyproline concentrations were used to calculate the actual hydroxyproline content for unknown samples. The factor 7.14 was then used to convert the hydroxyproline content to collagen content as hydroxyproline constitutes approximately 14% of the amino acids in Type I collagen (Etherington and Sims, 1981).

3.3.6 Ehrlich Chromogen quantification in SM

The concentration of the mature cross-link Ehrlich chromogen in lyophilized SM IMCT was determined by tryptic digest according to the method described by Horgan et al. (1991). An amount of 0.1 ± 0.003g of lyophilized IMCT was suspended overnight in 5 mL of 50 mM Tris-HCL containing 1 mM calcium chloride in a glass tube at 4 °C. The suspension was then heated

in a water bath at 65 ℃ for 1 hour, during which time each was vortexed at 0, 15, 30 and 45 minutes. The tubes were placed in another water bath at 37 ℃ to allow them to equilibrate and then 125 µL of trypsin solution was added to the suspension (0.5 mg trypsin/mL of sample solution) and incubated for 4 hours at 37 ℃. The trypsin solution was prepared by dissolving 20 mg of TPCK treated trypsin in 1 mL of 50 mM Tris-HCl buffer. The suspension was vortexed every 15 minutes during incubation. Following incubation, the trypsin digestion was heated again for 20 minutes at 65 ℃ to activate the enzyme trypsin and allowed to cool for 20 minutes to room temperature (25 ℃) to deactivate the enzyme. The suspension was then centrifuged at 28,000 g for 10 minutes and the supernatant was filtered through a 0.45 µm nylon filter into 5 mL Eppendorf tubes. Following filtration, 200 µL of 5% p-dimethyl-amino-benzaldehyde in 4 M perchloric acid, containing 0.01% mercuric chloride were added to 1 mL of filtrate in 1.5 mL Eppendorf tubes and centrifuged for 2 minutes at 14,000 g to remove turbidity. As quickly as possible (not exceeding 10 minutes after centrifugation), the absorbance of 1 mL of the supernatant was measured at 572 and 640nm. For each sample, the reading at the latter wavelength was considered the baseline measurement and was therefore subtracted from the former reading. The concentration of the pyrrole crosslink was calculated using a molar extinction coefficient of 25000 (Kemp and Scott, 1988) and expressed as mol/mol collagen and nmol/g raw muscle as shown below.

A/ε = molar concentration, where A = absorbance and ε = the molar extinction coefficient.

The remaining filtrate was kept at -20 ℃ for the determination of the total collagen content of the tryptic digest. This was done in duplicate as described above for hydroxyproline determination. In this determination however, as the supernatant was highly concentrated, 200 µL was diluted with 800 µL of deionized water to make the required 1 mL of sample.

3.3.7 Pyridinoline quantification in SM

For the quantification of PYR in IMCT, 0.2 ± 0.003 g of SM IMCT samples in duplicate were hydrolyzed in 6 mL of 6M HCl for 20 hours at 110 ℃ in a Teflon® capped glass tube flushed with nitrogen gas. After samples were hydrolyzed, they were cooled to room temperature and the hydrolysate was filtered using Whatman No. 4 filter paper (Fisher Scientific, Mississauga, Ontario). The tube and funnel were each rinsed with 1 mL of deionized water and also filtered. Out of the final filtrate volume of 8 mL, an aliquot of 0.5 mL was used for the quantification of total collagen through estimation of total hydroxyproline content as already described. The

remainder of the filtrate (7.5 mL) was used for the quantification of PYR crosslinks. The determination of PYR content was performed using the method described by Robins et al. (1996) while ensuring minimal exposure of IMCT and PYR hydrolysate to light (Sakura *et al.,* 1982). The 7.5 mL remainder of the filtrate from the acid hydrolysate was evaporated to dryness using a rotary evaporator at a temperature of 40 °C and 690 mm Hg (Heidolph Collegiate rotary evaporator, Brinkmann, equipped with a DistiVac Ultra auto-purge vacuum system, Brinkmann, Mississauga, ON) and reconstituted with 1.5 mL of 10% (v/v) acetic acid. PYR was separated by gel filtration (size exclusion chromatography) using polyacrylamide gel (Bio-gel P2, Bio-Rad, Canada) in gravity columns (Econopac, 20 mL bed volumes, 1.5 cm x 12 cm column, Bio-Rad, USA), equilibrated with 10% (v/v) acetic acid. PYR elution was performed with 10% (v/v) acetic acid. Fractions of 6 mL of PYR crosslinks were collected into 15 mL plastic graduated tubes (Thermofisher, Korea). The presence of PYR crosslinks was confirmed by measuring its content in 1 mL of each 5 mL fraction collected, based on its natural fluorescence with excitation at (λex) 295 nm and emission at (λem) 395 nm, using a fluorescence spectrophotometer (Lumina, Thermofisher, USA) (Robins *et al.,* 1996). Fractions confirmed to contain PYR cross-links were pooled, rotary-evaporated, and reconstituted with 1.5 mL of 0.1 M HCl (Fujimoto & Moriguchi, 1978). The reconstituted samples were further purified using cellulose phosphate cation-exchange column chromatography equilibrated with 0.1 M HCl. Fractions were collected as 6 mL fractions and the presence of PYR confirmed as described above. In between samples, columns were washed thoroughly with 0.1 M HCl to remove remaining amino acids and fluorophores. PYR was then eluted with 1 M HCl (Robins *et al*., 1996). Fractions confirmed to contain PYR were pooled and their volumes recorded. Pooled fractions were evaporated and reconstituted with 1.5 mL of 1% aqueous heptafluorobutyric acid (HFBA) (Sigma-Aldrich Canada, Ltd) and filtered (Acrodisc LC 13mm syringe filter with 0.2 µm PVDF membrane, Life Sciences) for quantification using reversed-phase high performance liquid chromatography (HPLC).

Reversed-phase HPLC separation of PYR crosslinks was performed at room temperature using a C18 column (Agilent Eclipse, XDB-C18, 5 µm, 4.6 × 150 mm) fitted with a pre-column filter (Isolation Technologies Precolumn Filter, 0.5um frit, Sigma-Aldrich, St. Louis, MI). The column was eluted using a binary solvent system where Solvent A was 0.13% HFBA in 22% methanol and Solvent B was 0.10% HFBA in 75% acetonitrile. The elution conditions (1.0 mL/min) were:0–10 min, 100% solvent A (washing); 10–12 min, 70% solvent A and 30% solvent

B (elution of pyridoxine, pyridoxamine standards); 12–14 min, 64% solvent A and 36% solvent B (elution of PYR), and 14–19 min, 100% solvent B (equilibration). Gradients were controlled using a pump equipped with an auto-sampler (Agilent 1200 HPLC, California, United States of America). Fluorescence of the eluted peaks was monitored at λex = 296 nm and λem = 400 nm (Agilent 1200 FLD, California, United States of America) and data were acquired electronically (OpenLab CDS, Agilent Technologies, California, United States of America). The PYR peak was quantified relative to a calibration curve prepared with a commercial PYR standard (Wako Chemicals USA). Molar PYR content in perimysium IMCT was calculated using a molecular weight of 429 g PYR/mol. Pyridinoline quantifications were performed in duplicate.

3.3.8 Statistical analyses

Statistical analyses were performed using R Studio (Version 3.5.1). Data for these measurements were analyzed as a 2 x 2 x 2 factorial, where RFI, steroid and ractopamine and their interactions served as fixed sources of variation. Analyses of total collagen and collagen heat-solubility data were performed using a split-plot design with RFI, Steroid, Ractopamine and their interactions as fixed effects at the animal (whole plot) level, and Ageing, and its interactions as fixed effects at the muscle (split-plot) level. Data were blocked by kill (slaughter day) and animal ID was used as a random source of variation. For significant ($P \leq 0.05$) models, the means were compared using planned least squares means comparisons adjusted using Tukey's method. Pearson's correlation coefficient analysis was performed to identify linearity of relationships between collagen heat-solubility characteristics and growth performance characteristics, and between collagen heat-solubility characteristics and meat quality traits using the rcorr function in the Harrel Miscellaneous Package (Hmisc) package in R Studio (Version 4.0.2). Pearson correlation coefficients were considered for significance at $P < 0.05$ and with a Bonferroni correction applied. A Bonferroni correction was applied by dividing the alpha value of 0.05 by the number of variables in the analysis (Rice, 1989).

3.4 Results

3.4.1 Collagen characteristics of the *semimembranosus* (SM) muscle

As stated in the materials and methods section Chapter 2 of this thesis, one steer (of low RFI status, implanted and RH supplemented) was removed from the study due to laminitis. The collagen fractions quantified were heat-soluble, heat-insoluble, total collagen and percent collagen solubility

For the SM muscle, RFI status had no effect on the soluble and total collagen fractions ($P > 0.05$) (Table 3.1). An interaction effect was observed between RFI status, steroid treatment and ageing period for insoluble collagen content of the SM muscle, where mean insoluble collagen content in muscles from steers of low RFI status that were implanted was higher at 3 days *post-mortem* than at 12 days *post-mortem* ($P = 0.0285$) (Figure 3.1). Another interaction effect was observed for insoluble collagen content of the SM muscle that involved RFI status, RH supplementation and ageing period. In this interaction, insoluble collagen concentration in the muscles of steers with low RFI status that were not RH supplemented was higher at 3 days *post-mortem* than at 12 days *post-mortem* ($P = 0.0308$) (Figure 3.2).

For the percentage of collagen solubility in the SM, RFI status, steroid treatment and days of ageing interacted ($P = 0.0353$). This interaction indicated that muscle collagen solubility percentage was higher at 12 days *post-mortem* than at 3 days *post-mortem* for high RFI steers that were not implanted. The muscle collagen solubility of the high RFI, non-implanted steers was also greater than that of low RFI steers that were implanted with steroids (Figure 3.3).

Table 3.1 Effect of RFI status on heat solubility and total quantity of intramuscular collagen of the *semimembranosus* (SM) muscle (means and standard errors presented).

Measurements	**RFI Status**		***P* Value**[1]
	Low (n=26)	**High (n=21)**	
Soluble (mg soluble collagen/ g raw meat)	0.297 (0.024)	0.302 (0.027)	0.8891
Total collagen (mg collagen/ g raw meat)	2.28 (0.08)	2.41 (0.09)	0.2813

[1] Probability of the F test, with significance at $P \leq 0.05$

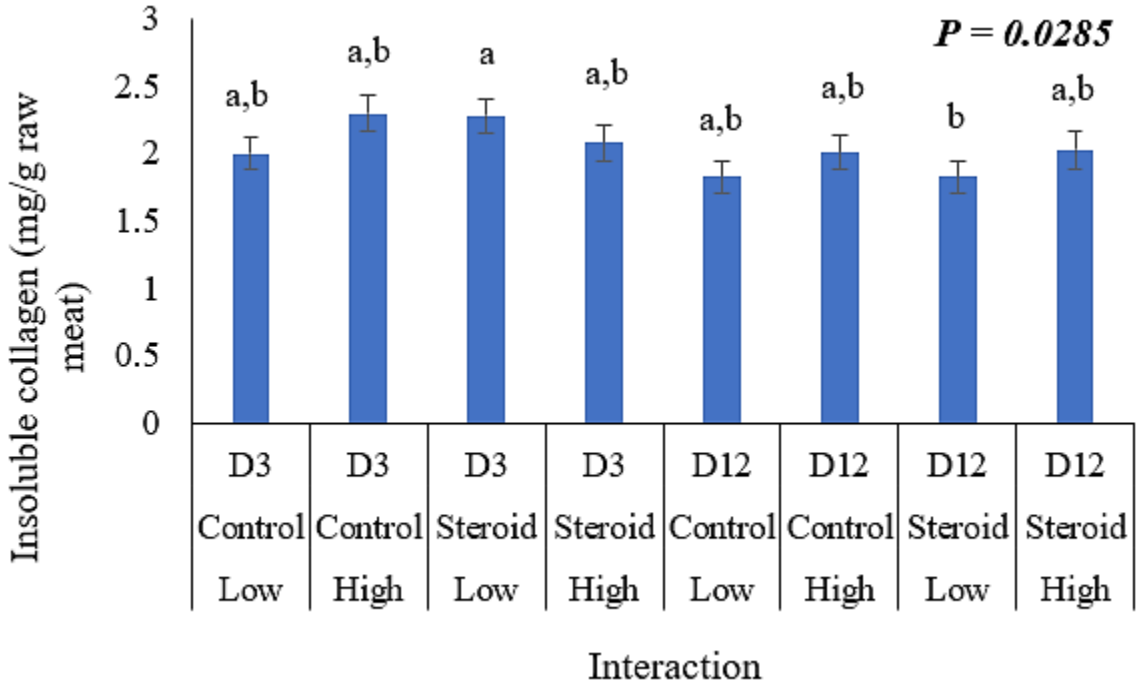

Figure 3.1 Insoluble collagen content of SM muscle samples from steers subjected to steroid treatment or not, and aged for 3 and 12 days *post-mortem*. X-axis legend first row: D3, 3 days post-mortem; D12, 12 days *post-mortem*. X-axis legend second row: control (no steroid) versus steroid (treated). X-axis legend third row: low versus high RFI. Treatment interaction means with different superscripts differ according to least square mean differences at $P<0.05$. Replication within day of ageing by treatment along the x axis was n = 13, 11, 13, and 10, respectively.

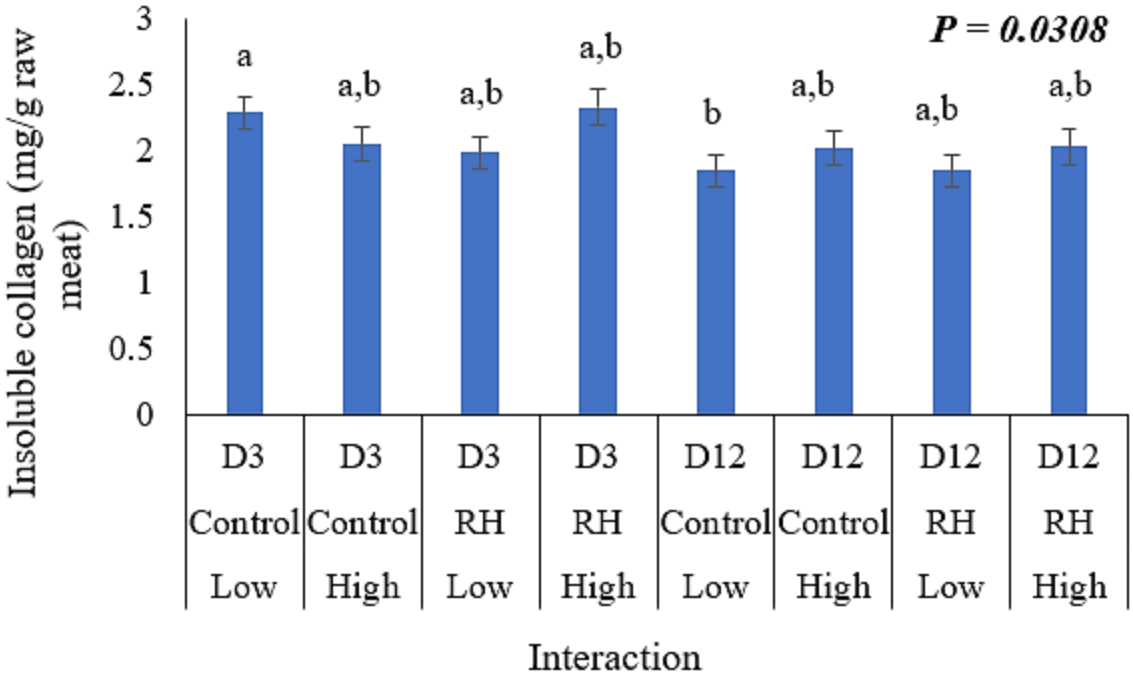

Figure 3.2 Insoluble collagen content of SM muscle samples from steers subjected to RH supplementation or not and aged for 3- and 12-days *post-mortem*. X-axis legend first row: D3, 3 days *post-mortem*; D12, 12 days *post-mortem*. X-axis legend second row: control (no RH) versus RH (treated). X-axis legend third row: low versus high RFI. Treatment interaction means with different superscripts differ according to least square mean differences at $P<0.05$. Replication for treatments was as follows: D3 and D12, non-RH supplemented, low RFI, n = 13; D3 and D12, non-RH supplemented, high RFI, n = 11; D3 and D12, RH supplemented, low RFI, n = 13; D3 and D12, RH supplemented, high RFI, n = 10.

Percentage collagen solubility in the SM was also influenced by an interaction between RFI status, RH supplementation and days of ageing (P = 0.0493). Collagen solubility was higher at 12 days than at 3 days *post-mortem* in SM muscles from steers of low RFI status that were not RH supplemented (Figure 3.4).

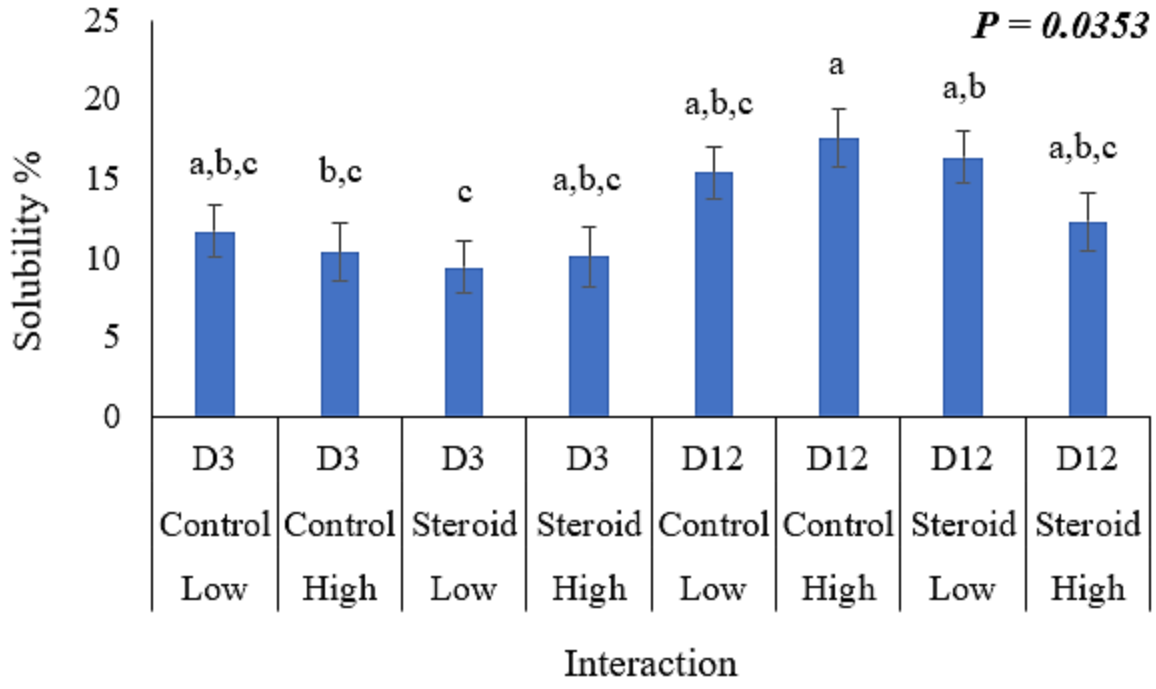

Figure 3.3 Percentage solubility content of SM muscle samples from steers subjected to steroid treatment or not, and aged for 3- and 12-days *post-mortem*. X-axis legend first row: D3, 3 days *post-mortem*; D12, 12 days *post-mortem*. X-axis legend second row: control (no steroid) versus steroid (treated). X-axis legend third row: low versus high RFI. Treatment interaction means with different superscripts differ according to least square mean differences at $P<0.05$. Treatment interaction means with different superscripts differ according to least square mean differences at $P<0.05$. Replication within day of ageing by treatment along the x axis was n = 13; 11, 13, 10, respectively.

Implantation with steroids had no effect on the soluble and total collagen characteristics measured in the SM muscle ($P > 0.05$) (Table 3.2). Steer supplementation with RH also did not influence the soluble and total collagen fractions measured in the SM muscle ($P > 0.05$) (Table 3.3).

Days of ageing had the greatest effect on the soluble collagen content of the SM muscle (Table 3.4), which was greater at 12 days *post-mortem* than at 3 days *post-mortem* ($P = 0.0001$).

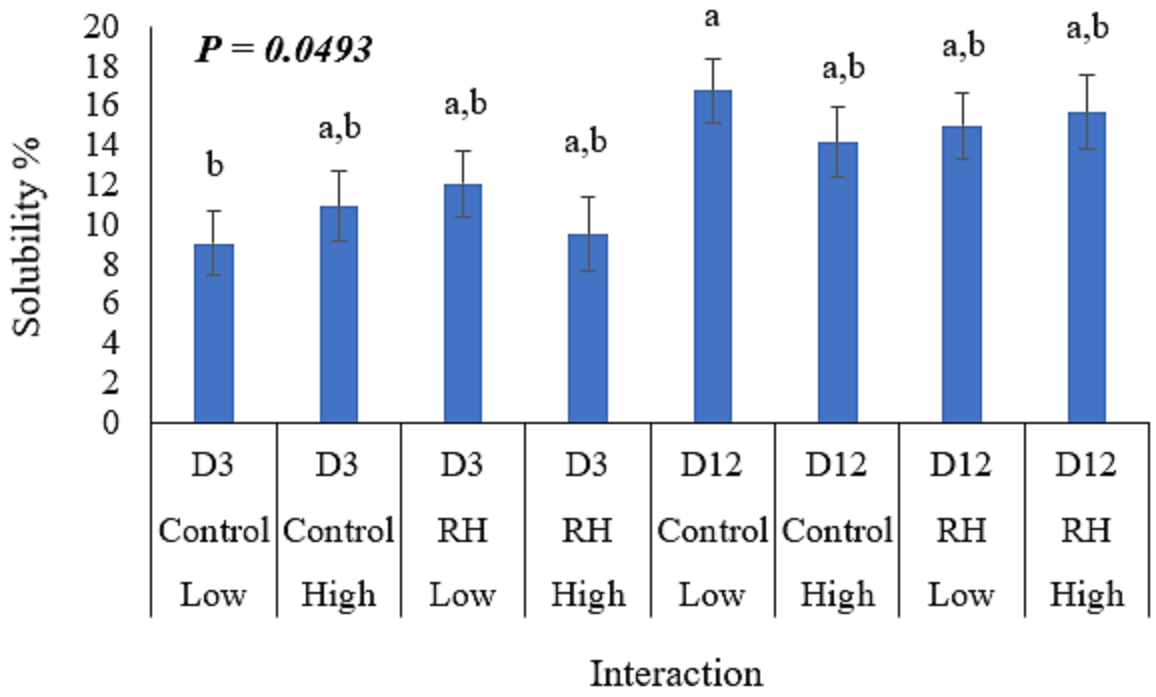

Figure 3.4 Percentage solubility content of SM muscle samples from steers subjected to selection for low RFI or not, RH supplementation or not, and aged for 3 or 12 days *post-mortem*. X-axis legend first row: D3, 3 days *post-mortem*; D12, 12 days *post-mortem*. X-axis legend second row: control (no RH) versus RH (treated). X-axis legend third row: low versus high RFI. Treatment interaction means with different superscripts differ according to least square mean differences at $P<0.05$. Replication for treatments was as follows: D3 and D12, non-RH supplemented, low RFI, n = 13; D3 and D12, non-RH supplemented, high RFI, n = 11; D3 and D12, RH supplemented, low RFI, n = 13; D3 and D12, RH supplemented, high RFI, n = 10.

Table 3.2 Effect of steroids on soluble and total collagen of the *semimembranosus* (SM) muscle (means and standard errors presented).

Measurements	**Steroid**		***P* Value**[1]
	Steroid (n=23)	**Control (n=24)**	
Soluble (mg soluble collagen/ g raw meat)	0.279 (0.026)	0.320 (0.025)	0.2542
Total collagen (mg collagen/ g raw meat)	2.33 (0.08)	2.36 (0.08)	0.8394

[1] Probability of the F test, with significance at $P \leq 0.05$

Table 3.3 Effect of RH supplementation on soluble and total collagen of the *semimembranosus* (SM) muscle (means and standard errors presented).

Measurements	Ractopamine hydrochloride		SEM	*P* Value
	RH (n=23)	Control (n=24)		
Soluble (mg soluble collagen/ g raw meat)	0.302	0.297	0.025	0.8994
Total collagen (mg collagen/ g raw meat)	2.34	2.35	0.08	0.9399

[1] Probability of the F test, with significance at $P \leq 0.05$

Table 3.4 Effect of days of ageing (DOA) on heat solubility of collagen of the *semimembranosus* (SM) muscle (means and standard errors presented).

Measurements	Days of Ageing		SEM	*P* Value
	Day 03 (n=47)	Day 12 (n=47)		
Soluble (mg soluble collagen/ g raw meat)	0.247[a]	0.352[b]	0.022	0.0001
Total collagen (mg collagen/ g raw meat)	2.41	2.28	0.07	0.0619

[a,b] Least square means within a row lacking a common letter differ at $P \leq 0.05$
[1] Probability of the F test, with significance at $P \leq 0.05$

3.4.2 Ehrlich's chromogen and pyridinoline cross-link concentrations and densities (mol EC/mol collagen) and total collagen (mg collagen/g raw meat) measured during quantitation of crosslinks from the *semimembranosus* (SM) muscle

The results showed no effect of RFI on perimysium collagen content (mg collagen/g raw meat) or on the densities and concentrations of Ehrlich's chromogen (mol EC/mol collagen and nmol EC/g raw meat) and pyridinoline (mol PYR/mol collagen and nmol PYR/g raw meat) ($P > 0.05$) (Table 3.5) except for a two-way interaction between RFI status and steroid treatment on total IMCT collagen content as measured during the assay for pyridinoline (mg collagen/g raw meat) ($P = 0.0222$). Although the interaction effect was significant, interaction means were not different according to least squares means differences. Numerical values indicated that the interaction arose from the application of steroids to low RFI steers increasing total SM collagen while it reduced total SM collagen in high RFI steers (Figure 3.5).

Steroid treatment did not influence SM muscle total perimysium collagen content (mg collagen/g raw meat) measured within the EC assay or Ehrlich's chromogen concentration (nmol EC/g raw meat) ($P > 0.05$) but decreased the Ehrlich's chromogen density (mol EC/mol collagen) ($P < 0.05$) (Table 3.6). Steroid implantation also increased the density of pyridinoline crosslinks in the SM (nmol PYR/g raw meat and mol PYR/mol collagen) ($P < 0.05$), however, with SM from implanted steers having similar concentration of collagen measured within the PYR assay as SM from non-implanted steers (Table 3.6).

RH supplementation increased the concentration of Ehrlich's Chromogen crosslinks in the muscle (nmol EC/g raw meat) ($P = 0.0262$) but this did not arise from an increased EC density in the collagen (Table 3.7). The effect of RH on total collagen estimated as part of the EC assay (mg collagen/g raw meat) approached significance with SM from RH-supplemented steers tending to have a higher mean total collagen content (nmol EC/g raw meat) than SM from steers not supplemented with RH ($P = 0.0785$) (Table 3.7). RH supplementation increased the density of PYR (mol PYR/mol collagen and nmol PYR/g raw meat) ($P < 0.05$) in the SM muscle. Furthermore, samples from RH supplemented steers had a higher total collagen content (mg collagen/g raw meat) than samples from non-supplemented steers ($P = 0.0123$) (Table 3.7).

Table 3.5 Effect of RFI status on cross-link density and total collagen content from IMCT of the *semimembranosus* SM muscle (means with standard errors in parentheses).

Measurements	**RFI**		***P* Value** [1]
	Low (n=26)	**High (n=21)**	
Ehrlich's chromogen density (mol EC/mol collagen)	6.50 (0.31)	6.62 (0.33)	0.7150
Ehrlich's chromogen concentration (nmol EC/g raw meat)	0.619 (0.015)	0.605 (0.014)	0.4960
Total IMCT collagen from Ehrlich's chromogen assay (mg collagen/g raw meat)	3.24 (0.14)	3.27 (0.15)	0.8348
Pyridinoline density (mol PYR/mol collagen)	0.216 (0.012)	0.226 (0.013)	0.4750
Pyridinoline concentration (nmol PYR/g raw meat)	3.51 (0.21)	3.78 (0.23)	0.2367
Total IMCT collagen from pyridinoline assay (mg collagen/g raw meat)	5.01 (0.20)	5.01 (0.21)	0.9833*

[1] Probability of the F test, with significance at $P \leq 0.05$
* Interaction between steroid and RFI treatment groups

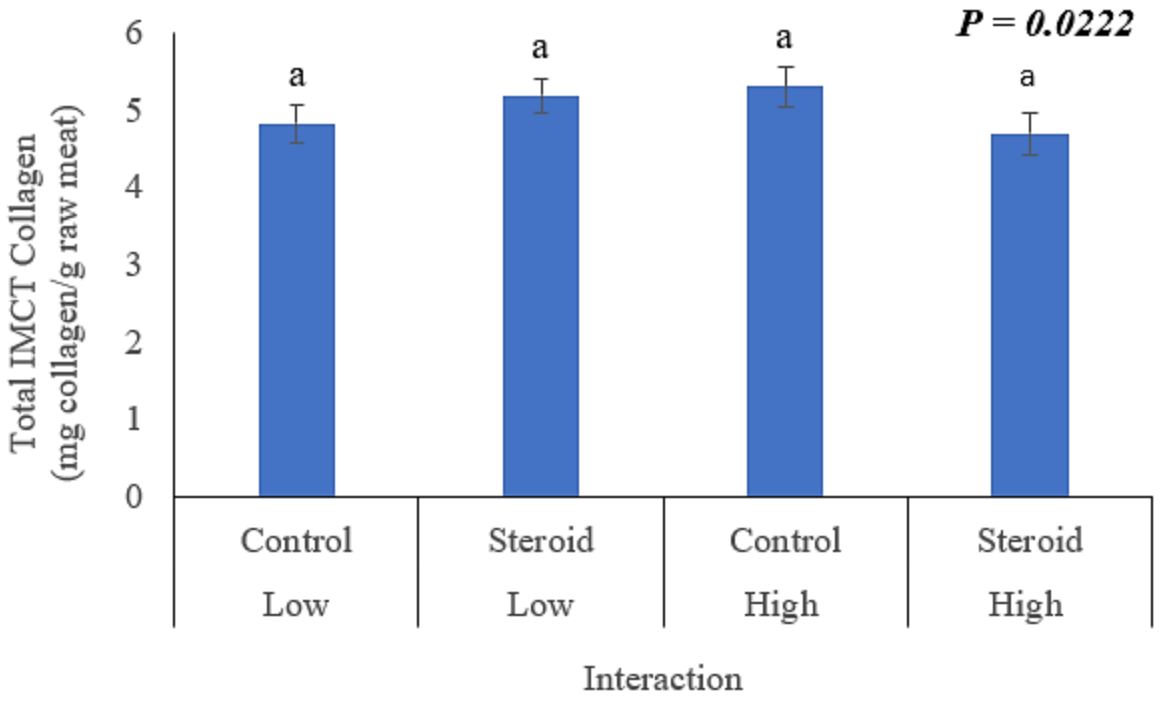

Figure 3.5 Total collagen values determined during pyridinoline analysis of IMCT samples from SM muscles from control and steroid-treated steers with low or high RFI status. X-axis legend: control versus steroid treatment; high versus low RFI. Replication for each treatment was: low RFI and control (non-implanted), n = 13; low RFI and implanted, n = 13; high RFI and control, n = 11; high RFI and implanted, n = 10. Treatment means with different superscripts differ according to Tukey's method for least square mean comparisons ($P < 0.05$).

Table 3.6 Effect of steroids on total collagen content and cross-link density from IMCT of the SM muscle (means with standard errors presented).

Measurements	Steroid (n=23)	Control (n=24)	*P* Value[1]
Ehrlich's chromogen crosslinks (mol EC/mol collagen)	0.589 (0.015)[a]	0.635 (0.015)[b]	0.0265
Ehrlich's chromogen crosslinks (nmol EC/g raw meat)	6.41 (0.32)	6.71 (0.32)	0.3066
Total collagen from Ehrlich's chromogen assay (IMCT) (mg collagen/g raw meat)	3.27 (0.15)	3.23 (0.14)	0.7956
Pyridinoline density (mol PYR/mol collagen)	0.239 (0.012)[a]	0.202 (0.012)[b]	0.0073
PYR concentration (nmol PYR/g raw meat)	3.87 (0.22)[a]	3.42 (0.22)[b]	0.0413
Total collagen from pyridinoline assay (IMCT) (mg collagen/g raw meat)	4.95 (0.20)	5.07 (0.19)	0.5588*

[a,b] Least square means within a row lacking a common letter differ at $P \leq 0.05$
[1] Probability of the F test, with significance at $P \leq 0.05$.
* Interaction between steroid and RFI treatment groups

Table 3.7 Effect of ractopamine hydrochloride (RH) supplementation on intramuscular connective tissue (IMCT) total collagen content and cross-link densities and concentrations in the SM muscle (means with standard errors presented).

Measurements	RH (n=23)	Control (n=24)	*P* Value[1]
Ehrlich's chromogen crosslinks (mol EC/mol collagen)	0.626 (0.015)	0.598 (0.15)	0.1645
Ehrlich's chromogen crosslinks (nmol EC/g raw meat)	6.89 (0.32)[a]	6.23 (0.32)[b]	0.0262
EC total collagen Ehrlich's chromogen assay (IMCT) (mg collagen/g raw meat)	3.37 (0.15)	3.13 (0.14)	0.0785
PYR crosslinks (mol PYR/mol collagen)	0.237 (0.012)[a]	0.204 (0.012)[b]	0.0161
PYR crosslinks (nmol PYR/g raw meat)	4.11 (0.22)[a]	3.17 (0.22)[b]	<0.0001
PYR total collagen pyridinoline assay (IMCT) (mg collagen/g raw meat)	5.28 (0.20)[a]	4.74 (0.20)[b]	0.0123

[a,b] Least square means within a row lacking a common letter differ at $P \leq 0.05$.
[1] Probability of the F test, with significance at $P \leq 0.05$.

Different estimates of total collagen concentration were obtained for the SM muscle from the three (3) different methods used to quantify total collagen, specifically the quantification of hydroxyproline from lyophilized muscle samples (HP), and from isolated IMCT used in the EC and PYR assays. The mean amount of total collagen from the lyophilized muscle was 2.42 ± 0.09 (mg collagen/ g raw meat), while that from isolated SM IMCT associated with quantification of EC and PYR were 3.26 ± 0.09 and 5.01 ± 0.09 mg collagen/ g raw meat, respectively.

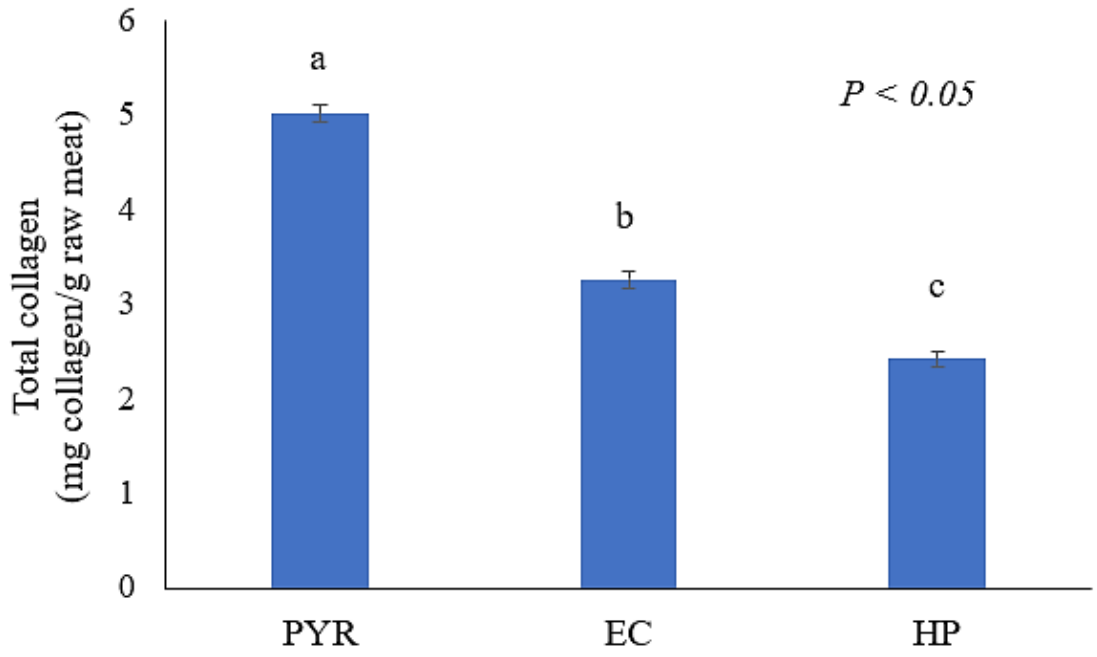

Figure 3.1 Total collagen concentration determined during pyridinoline (PYR) and Ehrlich's chromogen (EC) analyses from isolated IMCT, and from lyophilized meat samples (HP) respectively from SM muscles.

3.4.3 Pearson correlation coefficients between growth performance and collagen characteristics - SM

Pearson's correlation coefficients between live animal performance and collagen characteristics at day 3 *post-mortem* showed no significant relationships when the P value to which a Bonferroni correction had been applied was used ($P > 0.001$). At a significance of $P < 0.05$, however, pyridinoline (PYR2) (nmol EC/g raw meat) was lowly positively correlated with feed conversion efficiency during the second steroid period ($r = 0.29$) (P = 0.0492).

A strong positive correlation was observed between soluble collagen and collagen solubility at day 3 *post-mortem* ($P < 0.0001$) (Table 3.8). Total IMCT collagen from the Ehrlich's chromogen assay (Etotal) and the trivalent cross-link Ehrlich's Chromogen (EC1) (nmol EC/g raw meat) were highly positively correlated ($P < 0.0001$). A moderate positive correlation was found between total IMCT collagen from the PYR assay (PTotal) and pyridinoline (PYR2) (nmol EC/g raw meat ($P = 0.0008$) (Table 3.8). At a significance of $P < 0.05$, a negative moderate correlation existed between insoluble collagen and solubility ($P = 0.0065$) (Table 3.8). Total collagen from Ehrlich's Chromogen assay (Etotal) had a moderate positive correlation with collagen solubility ($P = 0.0206$), and a low positive correlation with soluble collagen ($P = 0.0494$) from lyophilized meat sample. A moderate positive correlation was found between the soluble collagen of lyophilized meat samples and EC1 ($P = 0.0162$), while a moderate positive correlation was found between collagen solubility of lyophilized meat samples and EC1 ($P = 0.0148$) (Table 3.8). Correlation results between growth performance and SM collagen characteristics showed no significant relationships ($P > 0.001$) at day 12 *post-mortem*. At $P < 0.05$, however, total collagen was moderately positively correlated with RFI status ($r = 0.31$) ($P = 0.0362$).

Table 3.8 Pearson correlations coefficients between collagen characteristics of the *semimembranosus* (SM) muscle samples aged for 3 days.

	ETotal[1]	PYR1[2]	PYR2[3]	EC1[4]	EC2[5]	Soluble	Insoluble	Total	Solubility
PTotal	0.53	-0.13	**0.47***	0.76	0.39	0.27	0.06	0.12	0.17
Etotal		0.08	0.39	**0.79***	-0.15	**0.29***	-0.21	-0.14	**0.34***
PYR1			0.80	0.04	0.05	-0.01	-0.06	-0.06	0.04
PYR2				0.49	0.29	0.15	-0.04	0.00	0.16
EC1					0.44	**0.35***	-0.08	0.00	**0.35***
EC2						0.24	0.17	0.21	0.17
Soluble							0.05	0.27	**0.88***
Insoluble								0.97	**-0.39***
Total									-0.18

*Asterisks indicate significance at $P < 0.006$.
[*]PTotal: Total IMCT collagen (mg collagen/g raw meat) quantified in association with pyridinoline
[1]ETotal: Total IMCT collagen (mg collagen/g raw meat) quantified in association with Ehrlich's chromogen
[2]PYR: Density of the trivalent crosslink pyridinoline (mol PYR/mol collagen)
[3]PYR2: Density of the trivalent crosslink pyridinoline (nmol PYR/g raw meat)

A moderate positive correlation was found between soluble collagen and total collagen of the SM muscle at day 12 *post-mortem* approached significance when the Bonferroni correction was applied ($P = 0.0029$) (Table 3.9). Total and insoluble collagen contents were strongly positively correlated ($P < 0.0001$) at day 12 *post-mortem* (Table 3.9). When considered significant at $P < 0.05$, insoluble collagen was moderately negatively correlated with collagen solubility ($P = 0.0176$).

Table 3.9 Pearson correlations between collagen heat-solubility characteristics of the *semimembranosus* (SM) muscle samples aged for 12 days.

	Insoluble	Total	Solubility
Soluble	0.03	**0.43***	0.91
Insoluble		**0.92***	**-0.34***
Total			0.05

*Coefficients bolded with asterisks indicate significance at $P < 0.05$.

There were no significant correlations between meat quality and collagen characteristics of the SM muscle at day 3 *post-mortem* ($P > 0.002$) (Table 3.10). With significance at $P < 0.05$, total collagen from lyophilized meat samples (Total) had a moderate positive correlation with pH ($P = 0.0340$), and a low positive correlation with fat content ($P = 0.0484$) at day 3 *post-mortem.* Total collagen from IMCT (Etotal) was moderately positively correlated with fat content ($r = 0.31$) ($P = 0.0361$) (Table 3.10). A low negative correlation ($P = 0.0465$) existed between PYR1 and drip loss, while a moderate negative correlation ($P = 0.0314$) was found between PYR2 and drip loss. EC2 and the colour value *L* were moderately negatively correlated ($P = 0.0319$). A low positive correlation was found between PYR1 and WBS ($P = 0.0614$), while a low negative correlation was found between EC2 and WBSF ($P = 0.0658$), which approached significance day 3 *post-mortem* (Table 3.10).

For the SM muscles aged for 12 days *post-mortem*, no significant correlations between meat quality and collagen characteristics were observed ($P > 0.003$) (Table 3.11). With significance at $P < 0.05$, soluble collagen was moderately positively correlated with the colour value *b** ($P = 0.0414$), while collagen solubility was moderately negatively correlated with WBSF at day 12 ($P = 0.0235$), lowly positively correlated with cook loss ($P = 0.0445$), and moderately positively correlated with the colour values hue ($P = 0.0123$) and *b** respectively ($P = 0.0099$) (Table 3.11).

Table 3.10 Pearson correlations between meat quality and collagen heat-solubility characteristics of the *semimembranosus* (SM) muscle samples aged for 3 days.

	Soluble	Insoluble	Total	Solubility	PTotal[1]	Etotal[2]	PYR1[3]	PYR2[4]	EC1[5]	EC2[6]
pH	0.17	0.28	**0.31***	0.04	0.02	-0.09	-0.21	-0.17	-0.13	-0.02
Temperature (°C)	-0.01	-0.09	-0.09	0.08	0.18	0.13	0.12	0.23	0.12	-0.04
Purge loss	0.13	-0.18	-0.14	0.20	0.01	0.19	0.04	0.06	-0.03	-0.17
Drip loss	-0.23	0.09	0.04	-0.25	-0.08	-0.19	**-0.29***	**-0.31***	-0.14	-0.004
L	-0.25	-0.12	-0.17	-0.19	-0.11	-0.16	-0.01	-0.12	-0.25	**-0.31***
*a**	-0.08	-0.12	-0.14	0.04	-0.18	0.00	0.08	-0.06	-0.11	-0.19
*b**	0.08	-0.10	-0.08	0.14	0.09	0.04	-0.10	-0.07	-0.05	-0.21
Chrome	-0.06	-0.10	-0.11	0.03	-0.10	0.03	0.06	-0.03	-0.10	-0.21
Hue	0.12	-0.08	-0.05	0.15	0.17	0.06	-0.14	-0.05	-0.01	-0.17
Cook loss	0.04	0.00	0.01	0.06	-0.12	0.00	0.22	0.16	0.00	0.08
WBSF (N)	-0.06	0.13	0.11	-0.12	-0.15	-0.02	**0.28***	0.12	-0.16	**-0.27***
Protein	-0.04	0.26	0.24	-0.19	0.08	-0.20	-0.15	-0.10	-0.22	-0.05
Ash	0.08	-0.10	-0.08	0.13	0.05	-0.12	-0.02	0.02	-0.08	0.02
Fat	0.24	0.24	**0.29***	0.08	0.22	**0.31***	-0.24	-0.10	0.25	-0.05

*Asterisks indicate significance at $P < 0.002$

[1]PTotal: Total IMCT collagen (mg collagen/g raw meat) quantified in association with pyridinoline

[2]ETotal: Total IMCT collagen (mg collagen/g raw meat) quantified in association with Ehrlich's chromogen

[3]PYR1: Density of the trivalent crosslink pyridinoline (mol PYR/mol collagen)

[4]PYR2: Density of the trivalent crosslink pyridinoline (nmol PYR/g raw meat)

[5]EC1: Density of the trivalent crosslink Ehrlich's chromogen (mol EC/mol collagen)

[6]EC2: Density of the trivalent crosslink Ehrlich's chromogen (nmol EC/g raw meat)

Table 3.11 Pearson correlations between meat quality and collagen characteristics of the *semimembranosus* (SM) muscle samples aged for 12 days.

	Soluble	Insoluble	Total	Solubility
pH	-0.02	-0.04	-0.05	0.04
Temperature (°C)	0.22	-0.22	-0.11	0.21
Purge loss	-0.17	0.10	0.01	-0.15
Driploss	-0.08	-0.07	-0.09	-0.05
*L**	0.06	-0.23	-0.19	0.11
*a**	0.20	-0.07	0.02	0.21
*b**	**0.30***	-0.24	-0.10	**0.37***
Chrome	0.23	-0.08	0.02	0.25
Hue	0.28	-0.26	-0.12	**0.36***
Cook loss	0.22	-0.25	-0.14	**0.29***
WBSF (N)	-0.27	0.12	0.002	**-0.33***

*Asterisks indicate significance at $P < 0.003$

3.4.4 Collagen characteristics of the *gluteus medius* (GM) muscle

For the GM muscle, RFI status did not influence the collagen characteristics measured ($P > 0.05$) (Table 3.14). Treatment with steroids influenced the insoluble collagen measured ($P = 0.0331$), where it was increased in muscles from non-implanted steers (Table 3.15). RH did not influence the collagen characteristics measured ($P > 0.05$) (Table 3.15). Decrease in total collagen in muscles from steroid treated steers approached significance ($P = 0.0559$).

No tendencies were observed. Days of ageing (DOA) had the greatest effect on the collagen characteristics measured in the GM muscle (Table 3.17). Soluble collagen content in GM muscles after 3 days of ageing was lower than that after 12 days of ageing ($P = 0.0110$). Insoluble collagen content was higher at day 12 of ageing than at day 3 of ageing ($P = 0.0093$) (Table 3.17). Mean total collagen content was significantly different in GM muscle samples aged for the different periods, with total collagen content from samples aged for 12 days was higher than the total collagen content from samples aged for 3 days ($P = 0.0039$). Solubility percentage was also increased in GM sample aged for 12 days ($P = 0.0047$) (Table 3.17).

Table 3.12 Effect of RFI status on collagen characteristics of the gluteus medius (GM) muscle.

Measurements	**RFI**		***P* Value**[1]
	Low (n=25)	**High (n=21)**	
Soluble (mg soluble collagen/ g raw meat)	0.999 (0.19)	0.713 (0.18)	0.2308
Insoluble (mg insoluble collagen/ g raw meat)	2.17 (0.18)	2.18 (0.19)	0.9493
Total collagen (mg collagen/ g raw meat)	3.17 (0.33)	2.90 (0.34)	0.4967
Solubility %	24.90 (2.88)	21.5 (3.04)	0.3195

[1] Probability of the F test, with significance at $P \leq 0.05$

Table 3.13 Effect of steroids on collagen characteristics of the *gluteus medius* (GM) muscle (means and standard errors presented).

Measurements	**Steroid**		***P* Value[1]**
	Steroid (n=23)	**Control (n=23)**	
Soluble (mg soluble collagen/ g raw meat)	0.999 (0.18)	0.713 (0.18)	0.2253
Insoluble (mg insoluble collagen/ g raw meat)	1.98 (0.18)[a]	2.37 (0.18)[b]	0.0331
Total collagen (mg collagen/ g raw meat)	2.70 (0.34)	3.37 (0.34)	0.0559
Solubility %	22.60 (2.94)	23.80 (2.93)	0.7040

[1] Probability of the F test, with significance at $P \leq 0.05$

Table 3.14 Effect of RH on the collagen characteristics of the *gluteus medius* (GM) muscle (means and standard errors presented).

Measurements	**Ractopamine**		***P* Value[1]**
	RH (n=22)	**Control (n=24)**	
Soluble (mg soluble collagen/ g raw meat)	0.795 (0.18)	0.917 (0.18)	0.6040
Insoluble (mg insoluble collagen/ g raw meat)	2.25 (0.18)	2.10 (0.18)	0.4073
Total collagen (mg collagen/ g raw meat)	3.05 (0.34)	3.02 (0.33)	0.9214
Solubility %	22.30	24.10	0.5752

	(2.97)	(2.89)

[1] Probability of the F test, with significance at $P \leq 0.05$

Table 3.15 Effect of days of ageing on the collagen characteristics of the *gluteus medius* (GM) muscle (means with standard errors in parentheses).

Measurements	**Days of Ageing**		***P* Value[1]**
	3 days (n=46)	**12 days (n=46)**	
Soluble (mg soluble collagen/ g raw meat)	0.590 (0.17)[a]	1.120 (0.17)[b]	0.0110
Insoluble (mg insoluble collagen/ g raw meat)	1.94 (0.18)[a]	2.42 (0.18)[b]	0.0093
Total collagen (mg collagen/ g raw meat)	2.53[a] (0.34)	3.54[b] (0.34)	0.0039
Solubility %	18.80 (2.89)[a]	27.60 (2.89)[b]	0.0047

[a,b] Least square means within a row lacking a common letter differ at $P \leq 0.05$
[1] Probability of the F test, with significance at $P \leq 0.05$

3.4.5 Pearson correlation coefficients between growth performance and collagen characteristics - GM

Pearson correlation coefficients analysis showed no significant relationships between live animal performance and intramuscular collagen characteristics of the GM muscle at 3 days *post-mortem* (P > 0.001) (Table 3.16). At a significance of $P < 0.05$, insoluble collagen was moderately positively correlated with the initial weight of steers before steroid treatment (WBS) ($P = 0.0279$) and had a low negative correlation with the average daily gain after the first steroid treatment from onset of study (ADGAFS) that approached significance ($P = 0.0545$). A low positive correlation was found between total collagen and WBS that approached significance ($P = 0.0589$). Collagen solubility had moderate positive correlations with average daily gain on entire study upon entry into Growsafe (ADGWSR) ($P = 0.0433$), feed intake on entire study (FIWSR) ($P = 0.0326$),

average daily feed intake after the first steroid period (ADFIFS) (P = 0.0351), average daily feed intake after the second steroid period (ADFISS) (P = 0.0335) and average daily feed intake of the entire study (ADFIWS) (P = 0.0343) (Table 3.16).

Table 3.16 Pearson correlations between growth performance and collagen characteristics of the *gluteus medius* (GM) aged for 3 days.

Measurement	Soluble	Insoluble	Total	Solubility
RFI	0.05	-0.05	0.002	0.18
WBS	0.18	**0.32***	**0.28***	0.25
GSW	0.03	0.14	0.10	0.22
WAFS	0.05	-0.01	0.02	0.28
WASS	-0.02	-0.02	-0.02	0.24
ADGRWSR	-0.13	-0.25	-0.21	0.14
ADGWSR	0.08	-0.18	-0.06	**0.31***
FIWSR	0.11	0.02	0.07	**0.32***
FEWSR	0.06	-0.22	-0.10	0.19
RHI	-0.19	-0.17	-0.20	0.01
RH4	-0.19	-0.24	-0.24	0.03
FIR	0.03	-0.14	-0.06	0.23
ADGR	-0.10	-0.21	-0.17	0.05
FER	-0.03	-0.06	-0.05	-0.03
ADFIFS	0.10	-0.02	0.04	**0.32***
ADGFS	0.09	-0.24	-0.09	0.16
FEFS	0.06	-0.22	-0.09	0.04
ADFISS	0.11	-0.03	0.05	**0.32***
ADGSS	-0.09	-0.04	-0.07	0.11
FESS	-0.09	0.04	-0.03	0.03
ADGWS	0.06	-0.14	-0.05	0.28
ADFIWS	0.10	-0.02	0.05	**0.32***
ADGRWS	-0.13	-0.21	-0.19	0.14
Feed conversion efficiency on combined steroid (FEWS)	-0.004	-0.17	-0.11	0.12
ADG on first steroid from onset of study (ADGAFS)	-0.10	**-0.29***	-0.22	0.10
Insulin-like growth factor 1 (IGF-1)	-0.02	0.03	0.01	-0.08
IGF Binding protein 3 (IGFBP3)	-0.004	0.01	0.01	-0.19
Slaughter weight (SW)	-0.19	-0.24	-0.24	0.03
Carcass weight (CW)	-0.21	-0.10	-0.17	-0.08

*Asterisks indicate significance at $P < 0.0015$.

RFI-Residual Feed Intake, WBS-Weight before steroid, GSW-Weight on entering Growsafe, WAFS-Weight after first steroid, WASS-Weight after second steroid, ADGRWSR-ADG on entire study at onset of steroid, ADGWSR-ADG on entire study from Growsafe entry, FIWSR-Feed intake on entire study, Feed conversion efficiency on entire study-FEWSR, RHI-Weight before RH supplementation, RH4-Weight after RH supplementation, FIR-ADFI on RH supplementation, ADGR-ADG on RH supplementation, FER-Feed conversion efficiency on RH supplementation, ADFIFS-ADFI on first steroid, ADGFS-ADG on first steroid treatment, FEFS-Feed conversion efficiency on first steroid, FESS-Feed conversion efficiency on second steroid, ADGWS-ADG on combined steroid, ADFIWS-ADFI on combined steroid, ADGRWS-ADG on entire study from onset of steroid, FEWS-Feed conversion efficiency on combined steroid, ADGAFS-ADG on first steroid from onset of study, IGF-1- Insulin-like growth factor 1, IGFBP3-IGF Binding protein 3, SW-Slaughter weight and CW-Carcass weight.

A moderate positive correlation was found between soluble and insoluble collagen content of the GM muscle at 3 days *post-mortem* ($P < 0.0001$) (Table 3.17). Soluble collagen content and solubility were strongly positively correlated at 3 days *post-mortem* ($P < 0.0001$). Strong positive correlations existed between total collagen content at 3 days *post-mortem* and soluble collagen and insoluble collagen ($P < 0.0001$), while a moderate positive correlation existed between total collagen and collagen solubility ($P < 0.0001$) (Table 3.17).

Table 3.17 Pearson correlations between collagen heat-solubility characteristics of the *gluteus medius* (GM) muscle samples aged for 3 days.

Measurement	Insoluble	Total	Solubility
Soluble collagen (mg soluble collagen/ g raw meat)	**0.62***	**0.90***	**0.83***
Insoluble collagen (mg soluble collagen/ g raw meat)		**0.90***	0.26
Total collagen (mg collagen/ g raw meat)			**0.60***

*Asterisks indicate significance at $P < 0.012$.

Pearson's correlation results showed no significant relationship between growth performance and collagen characteristics of the GM muscle at 12 days *post-mortem* ($P > 0.001$ and $P > 0.05$). Soluble collagen content and solubility were strongly positively correlated at 12 days *post-mortem* ($P < 0.0001$) (Table 3.18). Strong positive correlations existed between total collagen content at 12 days *post-mortem* and soluble collagen and insoluble collagen ($P < 0.0001$), while a moderate positive correlation existed between total collagen and collagen solubility ($P < 0.0001$). Soluble and insoluble collagen were moderately positively correlated ($P = 0.0005$) (Table 3.18).

Table 3.18 Pearson correlation coefficients between collagen characteristics of the *gluteus medius* (GM) muscle samples aged for 12 days.

Measurement	Insoluble	Total	Solubility
Soluble collagen (mg soluble collagen/ g raw meat)	**0.49***	**0.89***	**0.80***
Insoluble collagen (mg soluble collagen/ g raw meat)		**0.83***	0.08
Total collagen (mg collagen/ g raw meat)			**0.55***

*Asterisks indicate significance at $P < 0.012$.

Except for a moderate positive correlation between collagen solubility and muscle temperature at day 3 *post-mortem,* GM meat quality and other collagen characteristics were not correlated ($P > 0.003$) (Table 3.19) although correlations were found between the collagen characteristics. Although a Bonferroni correction was applied to the Pearson correlation coefficients as an indicator of significance, at a less stringent significance of $P < 0.05$, soluble, insoluble and total collagen all had moderate positive correlations with fat content ($P = 0.0091$, 0.0095, 0.0035 respectively) (Table 3.19). Insoluble collagen was moderately positively correlated with ash ($P = 0.0312$) and protein ($P = 0.0026$) contents. Insoluble collagen was also moderately positively correlated with cook loss ($P = 0.0293$) and the colour value *b* ($P = 0.0436$). A moderate positive correlation was found between total collagen and fat content ($P = 0.0035$). Solubility at day 3 *post-mortem* of the GM muscle was moderately positively correlated with hue ($P = 0.0380$) (Table 3.19).

At day 12 *post-mortem,* correlations between meat quality and collagen characteristics of the GM muscle were not significant ($P > 0.003$) (Table 3.20). Again, at a significance of $P < 0.05$, insoluble collagen was moderately negatively correlated with cooking loss ($P = 0.0328$) and moderately positively correlated with the colour value *a** ($P = 0.0107$). Total collagen had a moderate positive correlation with the colour value *a** ($P = 0.0407$).

Table 3.19 Pearson correlation coefficients between meat quality and collagen characteristics for the *gluteus medius* (GM) aged 3 days *post-mortem*.

Measurement	Soluble	Insoluble	Total	Solubility
pH	0.17	0.11	0.16	0.15
Temperature	0.29	-0.06	0.13	**0.49***
Purge loss	-0.02	0.02	0.00	0.02
Drip loss	0.14	-0.05	0.05	0.10
L	-0.22	-0.05	-0.15	-0.10
*a**	0.10	0.25	0.20	0.02
*b**	0.21	**0.30***	0.28	0.25
Chrome	-0.01	0.08	0.04	-0.11
Hue	0.24	0.27	0.28	**0.31***
Cook loss	0.20	**0.32***	0.29	0.22
WBSF	-0.20	-0.02	-0.12	-0.11
Protein	0.04	**0.43***	0.26	-0.07
Ash	-0.11	**0.32***	0.12	-0.26
Fat	**0.38***	**0.38***	**0.42***	0.17

*Asterisks indicate significance at P < 0.003.

Table 3.20 Pearson correlation coefficients between meat quality and collagen characteristics of the *gluteus medius* (GM) aged for 12 days *post-mortem*.

Measurement	Soluble	Insoluble	Total	Solubility
Ph	-0.14	-0.04	-0.12	-0.05
Temperature	-0.004	0.05	0.02	0.05
Purge loss	-0.05	-0.28	-0.18	0.17
Drip loss	-0.09	-0.17	-0.15	0.04
*L**	0.23	0.14	0.22	0.14
*a**	0.18	**0.37***	**0.30***	0.07
*b**	0.01	-0.13	-0.07	0.10
Chrome	-0.07	-0.10	-0.09	-0.01
Hue	-0.10	-0.22	-0.18	-0.03
Cook loss	-0.10	**-0.32***	-0.23	-0.03
WBSF	0.23	0.15	0.23	0.11

*Asterisks indicate significance at *P* < 0.003.

3.5 Discussion

Connective tissue functions physiologically to provide support for myofibrillar tissue and further transmits the force produced by actin and myosin, also known as the contractile proteins, through tendons to the skeleton. This explains why the connective tissue is associated with toughness and resilience (Stanton and Light, 1987). The large IMCT structure, the perimysium, has been noted to contribute to meat toughness (Nishimura *et al.,* 1995, 1999; Nakamura *et al.,* 2003). Nakamura et al. (2003) showed that the total amount of collagen in the *pectoralis profundus* muscle (brisket) was related to its perimysium, which is well developed. These observations make the perimysium a crucial study to ascertain its effect on meat texture and consequently meat tenderness. *Post-mortem* conditioning, otherwise known as *post-mortem* ageing, improves the tenderness of beef within ageing periods ranging from 2-3 weeks in cold or refrigerated storage, during which the meat attains its peak eating quality. The network of contractile proteins (Locker 1960; Marsh and Leet, 1966) and collagen fibres remain the two leading contributors to meat toughness, with the collagen fibres providing what has become known as the background toughness of meat (Bailey, 1972).

According to Purslow (2005), it is accepted that collagen content has a significant influence on beef toughness of different muscles and represents the background toughness of meat after an extended ageing period. Total collagen content consists of the soluble and insoluble portions, where the soluble portion is most desired. Generally, none of the individual collagen characteristics are closely related to meat tenderness (Lepetit, 2007). In this thesis chapter, collagen characteristics of the *semimembranosus* (SM) and *gluteus medius* (GM) muscles were measured to elucidate the effect RFI status, growth promotants and *post-mortem* ageing, and their interactions, if any, on them. That the influence of production practices on collagen solubility was evident only in the SM implied that perturbation due to management practices is most evident in high connective tissue muscles like the SM. The results observed in this thesis chapter indicated that collagen solubility increased with *post-mortem* ageing in the SM muscle from steers when neither RFI selection nor steroids were applied, in those selected for low RFI that did not receive RH, or when steers were selected for low RFI and steroids were applied. These results are seemingly incongruent, as the former were shown to grow the slowest in Chapter 2 (Coleman *et al*., 2021), and would therefore be expected to exhibit evidence of MMP activity *post-mortem*,

while the latter two would be expected to have limited evidence of MMP activity and hence limited increase in collagen heat solubility due to possible increased inhibitor (TIMP) activity in response to selection for low RFI and growth promotion. The use of growth promotants has been reported to increase collagen contribution to beef toughness (Girard *et al.*, 2012), and decrease collagen heat solubility (Cranwell *et al*., 1996a; Cranwell *et al*., 1996b). Sylvestre et al. (2002) however found that increasing the growth rate of lambs increased MMP2 activity *post-mortem*. Drawing from the results of Sylvestre et al. (2002), slow growth may decrease the need for skeletal muscle collagen remodelling thus decreasing MMP activity ante- and *post-mortem*, but this was not further investigated in this thesis.

Also, in this thesis chapter, the density of the crosslinks PYR and EC were measured from isolated IMCT of the SM muscle, together with their associated perimysial collagen content. Steroid application increased total collagen as measured in the PYR assay in low RFI steers but decreased it in high RFI steers. Of the methods used in this thesis to estimate total muscle collagen, the one associated with the PYR assay is likely the most accurate because it estimates collagen in the isolated intramuscular connective tissue. Roy et al. (2021) acknowledged this in their report, indicating that grinding or blending of lyophilized muscle does not homogenize a sample due to the electrostatic attractions between the intramuscular collagen molecules. The means in this interaction were not significant, however, so this result only suggests that there is a tendency for intramuscular collagen content associated with the application of steroids to differ between high and low RFI steers. Selection for low RFI alone did not affect intramuscular collagen content, and this observation agrees with that of Zorzi et al. (2013). The effects of steroids on collagen are commonly known, but the mechanisms responsible for these effects are not well understood (LeBlanc *et al.,* 2017). The application of steroids, such as estrogen and testosterone, is common in human medicine to increase collagen and myofibrillar protein synthesis in injury or hormone deficiency situations (Hansen *et al.,* 2009, 2012; Jones *et al.,* 2018). Estrogen also increases collagen synthesis in post-menopausal women in response to exercise (Hansen *et al*., 2009) despite inhibiting lysyl oxidase (Lee *et al.,* 2015), while testosterone stimulates lysyl oxidase activity (Bronson et *al.*, 1987). Given that the steers in this study received a final implant primarily constituted of the testosterone analog trenbolone acetate, lysyl oxidase activity would have been anticipated to be increased in both low and high RFI steer SM. Why steroids would interact with

RFI status is not clear and warrants further research in a larger population of cattle to determine if this is a type II statistical error.

Implantation of steers with steroids had a clear effect on trivalent collagen cross-link concentrations and densities, decreasing EC density (mol EC/mol collagen) and increased PYR density and concentration in the SM muscle. PYR and EC cross-link densities and intramuscular concentrations in this thesis were within the expected range for bovine muscles. According to Bosselmann et al. (1995), PYR densities reported for three different muscles from heifers, steers and bulls ranged from 0.04 to 0.28 (mol/mol collagen). Similarly, PYR densities and concentrations reported by Roy et al. (2015) ranged from 0.17 to 0.21 (mol PYR/mol collagen) and 2.59 and 2.76 (mg PYR/ g raw muscle), respectively, in the *semitendinosus* and *gluteus medius* muscles and were also comparable to PYR results in this thesis. Roy et al. (2015) also reported densities and concentrations of EC between 0.45 to 0.56 (mol EC/mol collagen) and 10.56 to 10.95 (nmol/g raw meat), respectively, in the *semitendinosus* muscle which were also comparable to results found in this thesis.

Mature or trivalent crosslinks connect collagen molecules by providing linkages between collagen molecules via the telopeptide and helical regions of adjacent collagen molecules (Eyre, 1987). Although EC density was decreased with steroid application, the decrease was not of sufficient magnitude to decrease the overall concentration of EC in the muscle. The increased concentration of PYR in the SM with steroid application arose from both an increased density of PYR in collagen and an increase in total intramuscular collagen. These results do not agree with results obtained from the study by Roy et al. (2015) where implantation significantly increased EC densities in GM collagen (mol EC/mol collagen), and in raw GM muscle (mg collagen/g raw meat. This dissimilarity in results may have been due to Roy et al. (2015) using the GM, and to their steers being 12 to 13 months old at harvest, while the steers studied in this thesis were about 16 to 18 months old, although Roy et al. (2021) found no difference in intramuscular EC concentration between steers from these ages. The increase in collagen and raw meat PYR density with steroid administration in the thesis, however, do agree with the results of Roy et al. (2015) where steroid administration increased the density of PYR in the *gluteus medius* and *semitendinosus* muscles. The SM results in this thesis contrast with the results of Ebarb et al. (2016, 2017) where the use of growth promotants had no impact on the trivalent cross-link PYR, but their results were for the *longissimus lumborum* muscle, generally considered to be a low collagen muscle involved in

limited movement. The response of the trivalent crosslinks PYR and EC to steroid treatment observed in this thesis corroborate observations by Gerrard et al. (1987) and Judge et al. (1984), who noted that reduced collagen solubility was associated with the use of steroid hormones in cattle, particularly testosterone, as decreased collagen heat-solubility is associated with increased PYR concentration (Horgan *et al*., 1991; Bosselmann et al., 1995).

The use of implants has been reported to increase insulin-like growth factor 1 (IGF-1) levels in vivo (Barengolts *et al.,* 1996; Johnson et al., 1998). Roy et al. (2015) hypothesized that MMP activity was decreased in steroid-treated steers, allowing for PYR to become established in intramuscular collagen with a lower turnover. IGF-1 has been implicated as being indirectly responsible for the increased synthesis of collagen (Raiser *et al.,* 2007) but has not been related to specific crosslink formation. Results from blood analyses of IGF-1 reported in Chapter 2 of this thesis also showed similar circulating IGF-1 levels among treated and control groups 30 days after implantation, despite implantation increasing final live and hot carcass weights of implanted steers. IGF-1 serum levels would be expected to be detectably greater than those of control steers (Johnson *et al.,* 1998) and accompanied by increased IGF-1 mRNA in muscle (Pampusch *et al*., 2003), and so why this did not occur in the cattle studied in this thesis is unclear.

RH supplementation also increased both intramuscular PYR and EC concentrations of the SM. The increase in the PYR crosslink density and concentration contrasts with the results of Roy et al. (2015), where supplementation with RH decreased the molar concentration of PYR (mol PYR/mol collagen) in the GM muscle and increased the EC density in the *semitendinosus* (ST). Roy et al. (2015) hypothesized that changes in intramuscular PYR, EC and collagen concentrations due to RH were associated with the TGF-β1 pathway, which would preferentially form PYR (Gjaltema *et al*., 2015) as observed in this thesis, while those provoked by steroid administration were linked to the growth hormone (GH) and IGF-1 axis. Briefly, continuous supplementation of RH has been observed to increase intracellular concentration of G-protein-linked receptor kinase 2 (GRK2) which leads to the phosphorylation of β-AA receptors (Lynch and Ryall, 2008). Phosphorylation of the receptor by GRK2 uncouples the receptor from the G-protein and additionally deactivates the production of cyclic-adenosine monophosphate (cAMP) by adenylyl cyclase, which leads to the reduction of level of cAMP, leading to further reactions involving the Smad3/4 complex through activation by TGF- β1, and finally results in the stimulation of collagen

synthesis. Among the numerous cytokines and growth factors that regulate fibrogenesis, transforming growth factor (TGF)-β is one of the most important (Liu and Pravia, 2010). TGF-β1, TGF-β2, and TGF-β3 are the three identified isoforms of TGF-β. While TGF-β1 is expressed primarily in endothelial cells, fibroblasts, hematopoietic cells, and smooth muscle cells, TGF-β2 is primarily expressed in neurons and epithelial cells. The isoform TGF-β3 is particularly expressed in mesenchymal cells (Ghosh *et al.,* 2005). According to Letterio and Roberts (1998), all TGF-β isoforms activate down-stream SMAD signalling. SMADs have been shown to regulate the expression of collagen in a steady state (Schnabl *et al.,* 2001). The receptor-regulated SMADs (RSMADs) are a part of the SMAD family and include SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8, which are downstream effectors of serine-threonine kinase receptors that are activated when bound to TGF-β (Ross and Hill, 2008). Two inhibitor SMADs, (I-SMAD 6 and SMAD 7) constitute the remainder of the SMAD family (Moustakas *et al.*, 2001). TGF-β, a ligand, initially binds TGF-β receptor II (TβRII), with TβRII then recruiting and activating TβRI. This results in the phosphorylation and subsequent binding of SMAD2 and SMAD3 to SMAD4 (Suwanabol *et al*., 2011). The SMAD complex formed is then translocated into the cell nucleus. In the nucleus, the SMAD complex binds to SMAD-specific binding elements on target collagen genes, leading to the activation of expression of genes involved in fibrogenesis. These fibrogenic genes include those for procollagens and enzymes that catalyze the formation of collagen crosslinks (Massague and Chen, 2000). Measurement of circulating TGF-β1 and muscle RNA expression of TGF-β1 is therefore recommended in future investigations to determine if TGF-β expression is affected locally rather than systemically by steroid application.

Although the deterministic pathway of collagen synthesis was not revealed by the IGF-1 results, the formation of PYR and EC appeared purposeful, and were most likely determined by the levels of expression of the three isoforms of lysyl hydroxylase (LH) in muscle fibroblasts (Bruce and Roy, 2019; Roy *et al*., 2021). Upregulation of LH1 would lead to the formation of both EC and PYR, while upregulation of LH2 would preferentially lead to the formation of PYR (Eyre and Wu, 2005; Yamuachi and Sricholpech, 2015). With this considered, although not measured in this thesis, LH2 would likely be the predominant LH in the muscle of steroid-treated steers as they showed an increase in PYR density and concentration and a decrease in EC density. Similarly, LH2 likely predominated in the muscles of RH-treated steers as well, given that PYR density increased in treated steers with no change in EC density, while both PYR and EC concentrations

increased in treated steers due to an increase in intramuscular collagen. The expression of the *LH2* gene is therefore warranted to ascertain its role in the formation of the trivalent crosslinks, particularly, PYR.

Days of ageing (DOA) in this thesis had the most significant effect on the collagen heat-solubility measures of both SM and GM muscles, particularly on the soluble collagen content and solubility percentage of both muscles. Differences in the total concentration of collagen in the muscles between ageing times indicated there were positional differences in the steaks used at each ageing time; therefore, collagen heat solubility was interpreted after adjustment to a percentage of total collagen. The percentage of collagen solubility increased with days of *post-mortem* ageing in the GM irrespective of production treatment, but that of the SM increased with *post-mortem* ageing in steers that had not been treated with steroids and not selected for low RFI and in those that had been selected for low RFI and received steroids. These results support there being enzymatic degradation of the collagen matrix, at least between the first 3- and 12-days *post-mortem*, confirming that collagen is not resistant to proteolysis during ageing of meat. Increases in collagen solubility associated with ageing are due to matrix metalloproteinases activity during the *post-mortem* period, as this family of enzymes cleaves collagen in the extracellular space (Wu *et al.,* 1991; Freije *et al*., 1994; Knauper *et al*., 1996) and are active *post-mortem* (Balcerzak *et al*., 2001; Sylvestre *et al.,* 2002).

According to Purslow (2005), the contribution of IMCT to the texture of meat has previously been thought to be immutable, with the background toughness of meat not expected to change even with *post-mortem* ageing. Early research on IMCT showed that collagen solubility was neither affected by temperature nor period of conditioning (Pierson and Fox, 1976; Chizzolini *et al*., 1977). Stanton and Light (1988, 1990), however, reported that collagen found in the perimysium of the IMCT was solubilized during *post-mortem* ageing, implying that collagen may in fact be solubilized during *post-mortem* ageing under certain circumstances. Earlier studies by Pierson and Fox (1976) and Chizollini et al. (1977) demonstrated that collagen solubility was affected by neither temperature nor conditioning period, which implied that collagen is not changed at the molecular level during ageing. According to Stanton and Light (1988; 1990) however there is evidence to dispute this concept. Stanton and Light (1988) highlighted in their study that the IMCT is altered during *post-mortem* ageing, where aged meat contained an amount of solubilized collagen in the perimysium that was significantly higher than unaged meat,

suggesting damage to collagen in the perimysium through *post-mortem* proteolysis, mainly by the action of cathepsins. This was observed using a two-dimensional SDS polyacrylamide gel electrophoresis, where a general diminishing of residual insoluble perimysial collagen was seen, as well as damage to specific peptides, and the appearance of new bands signifying the formation of other new peptides. Stanton and Light (1990) showed that soluble endomysium increased with *post-mortem* ageing. In this study, four different bovine muscles used in two groups (aged and unaged) were compared where three of the aged muscles had higher amounts of soluble endomysium compared to their unaged counterparts, the exception being the *supraspinatus* muscle. The concentration of insoluble endomysium was found to be lower in three of the aged muscles compared to the unaged muscles, the exception being the *gastrocnemius* muscle. The collagen content of the soluble fraction of the endomysium was found to be higher in aged than unaged muscles, in all four muscles used. The authors also showed that the ratio of collagen type I to III increased after ageing in the *gastrocnemius* and *psoas major* muscles, indicating a reduction in the amount of collagen type III upon *post-mortem* ageing. With the aid of a differential scanning calorimetry, however, Judge and Aberle (1982), showed that the thermal shrinkage temperature of bovine intramuscular collagen decreases by 7 - 8 °C within 7 days of *post-mortem* ageing. Also, Etherington (1987) reported that the isometric tension of the intramuscular collagen in beef decreases at 21 days *post-mortem*. Nishimura et al. (1998) reported that the shear-force value of raw beef decreased quickly up to 10 days, after which the decrease became gradual, reaching 56% of the initial shear-force value after 35 days of *post-mortem* ageing. The authors also reported that the shear-force value of raw IMCT did not change for up to 10 days *post-mortem* but decreased afterwards and that the yield (the total amount of hydroxyproline) of the fraction of perimysium, measured as an indicator of mechanical properties of the perimysium, did not change during a *post-mortem* period of 14 days, but subsequently decreased gradually. Ebarb et al. (2016) reported an increase in the amount of soluble collagen by 10% in raw *longissimus* lumborum muscles from day 2 to day 35 of *post-mortem* ageing. Jeremiah et al. (1981) also reported an increase by 12% in collagen solubility for *longissimus dorsi* muscle aged from 1 to 20 days. The results of this thesis, in conjunction with the most recent literature, substantiate and support increases in intramuscular collagen solubility with *post-mortem* ageing; therefore, *post-mortem* ageing is a critical step in enhancing beef tenderness in the modern beef supply chain.

Interestingly, there were few differences between day 3 and 12 percentages of soluble collagen across the treatments, suggesting that levels of collagen solubility at day 3 were already elevated in some treatments, with no evidence of MMP activity with time *post-mortem*. Our result contradicts results from a study by Zorzi et al. (2013) where low RFI Nellore bulls had a greater percentage of soluble collagen than their high RFI counterparts. Why there were few increases in collagen solubility with *post-mortem* ageing is unclear, but with growth there is turnover in the collagen network with lean or skeletal muscle deposition where newly synthesized collagen is deposited in muscle tissue to support muscle remodelling (Purslow, 2014). The increase in the rate of connective tissue turnover may reduce the rate of development of mature crosslinks, which are associated with meat toughness (Christensen and Purslow, 2016). The newly synthesized collagen, which is deemed immature, is made of divalent crosslinks that are heat labile, and hence soluble (Bailey and Light, 1989; McCormick, 2009). The resulting increase in collagen heat-solubility due to rapid growth in animals is most likely due to reduced cross-link valency associated with synthesis and remodelling of the connective tissue (McCormick, 1994). Increased synthesis of new collagen in cattle unselected for RFI receiving steroids and in those selected for RFI not receiving steroids may have elevated the percentage of soluble collagen at day 3 *post-mortem* at a time when the demands for IMCT growth reduced MMP expression. Several studies have shown a relationship between protein turnover and RFI (Archer *et al*., 1999; Basarab *et al.,* 2003, Robinson and Oddy, 2004). Data exist to support the hypothesis that variations in RFI are related to body protein gain and protein degradation (Richardson and Herd, 2004). According to Richardson et al. (2001), negative correlations exist between RFI and the percentage of body protein, as well as between RFI and protein gain, suggesting that animals of low RFI status have the capacity to preferably deposit protein more effectively, rather than degrade protein. This is an indication that low RFI animals have a lower rate of muscle protein turnover (Zorzi *et al.,* 2013), which may possibly and subsequently result in reduced MMP and increased TIMP activities. MMP and TIMP activities were not measured in this thesis, however, and further investigation into the expression of genes for proteins related to collagen synthesis and degradation is warranted to fully understand the effects of RFI selection and growth promotants on proteins related to collagen synthesis and degradation.

Pearson correlations in this thesis showed no significant relationships between WBSF, EC, PYR and collagen solubility in the SM, and no relationships between WBSF and collagen

solubility in the GM. However, with significance at $P < 0.05$, EC concentration was positively correlated with collagen solubility at day 3 *post-mortem*, and the correlation between PYR and EC densities and WBSF at day 3 approached significance, supporting findings that EC is not as heat stable as PYR (Horgan *et al.,* 1991; Roy *et al*., 2021). Also, at day 12 *post-mortem*, SM collagen solubility was negatively correlated with WBSF at day 12 when significance was at $P < 0.05$, suggesting that the relationship between collagen solubility and WBSF improved with *post-mortem* ageing. It is worth noting that correlations between the number of crosslinks per collagen molecule and meat tenderness range from relatively high (Bailey and Light, 1989) to low or non-significant values (Avery *et al.*, 1996) among different muscles. These results indicate that the contribution of collagen crosslinks to SM WBSF are greatest early *post-mortem* before substantial MMP activity would be anticipated. With the anticipated degradation of collagen by MMPs during the full *post-mortem* period, the relationship between collagen solubility and WBSF should become significant, and it did.

Further in the thesis, supplementation with RH did not influence the soluble and total collagen content. This agrees with results of Girard et al. (2011) who used samples from the *semitendinosus* and *gluteus medius* muscles and reported no effect of the use of implants and RH on the total and soluble collagen content of both muscles. Strydom et al. (2009), reported no difference in the total collagen content between RH supplemented and non-supplemented groups, which agrees with our results. A similar result was reported by Martin et al. (2014), where the total collagen content between RH supplemented steers and control steers was not significantly different. According to Gerken et al. (1995) and Strydom et al. (2009), the use of growth promotants, which include hormonal or synthetic steroids, negatively affect meat tenderness through the increased activity of calpastatin. From our understanding of the contribution of collagen to the background toughness of meat, it was expected that the use of growth promotants would result in significant differences in total collagen and collagen solubility in the SM and GM muscle between steers from treated and control groups. As significant differences were found in the WBSF of both GM and SM muscles between implanted and control steers (reported in the 2nd chapter of this thesis), we can conclude that the effect of growth promotants on meat tenderness is mostly limited to muscle fibres with the cooking methods used in this thesis.

3.6 Conclusions

This study confirmed that the use of steroids and RH in steers increased the concentration and density of intramuscular PYR in the SM without affecting the heat solubility of its collagen. This study also indicated that selection for low RFI did not affect collagen characteristics in either muscle studied. *Post-mortem* ageing, however, increased collagen solubility of both GM and SM muscles in beef steers not treated with growth promotants, confirming that collagen is not immutable to *post-mortem* ageing. Further studies and clarification on specific mechanisms by which the solubilization of IMCT collagen occurs during *post-mortem* ageing of muscles are needed to provide insight into the regulation of *post-mortem* tenderization of meat.

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Chapter 4. Profiling of the expression of genes related to connective tissue synthesis and degradation as affected by beef steer RFI status and growth promotant use and their relationship to beef quality in the *semimembranosus*

4.1 Introduction

The eating quality of beef is influenced by production factors such as animal breed, sex, slaughter age, housing, nutrition/feed as well as animal handling before slaughter (Page *et al*., 2011; Honkavaara *et al.,* 2003; Hoquette *et al.,* 2006; Weglarz, 2011). Other factors that affect meat quality characteristics occur *post-mortem* and these include post-slaughter processes such as chilling/storage temperature, ageing/conditioning, and packaging (Kołczak *et al.*, 2003; Brooks and Savell, 2004). Hormonal implants and beta-adrenergic agonists (β-AA) are known to redirect nutrients towards lean tissue deposition and away from adipose tissue. Johnson and Chung (2007) hypothesised that the use of growth promoters first affects the direction of specific non-differentiated "stem cell-like" mesodermal cells. When these cells differentiate towards a particular phenotype, it is often permanent and cannot be reversed.

In-depth knowledge of the cellular mechanisms influenced by growth promoters can potentially explain the re-direction or prioritization of nutrient use by the whole animal (Johnson and Chung, 2007). According to Cheng et al. (2020), although the genetic hierarchy of skeletal muscle formation has widely been studied, a major portion of the factors that regulate these processes remain unknown. This is due to the complication associated with transcriptional regulation of key genes during the development of the muscle. As such, more understanding of the transcriptional regulation during the development of the skeletal muscle is needed (Chen *et al*., 2020).

To meet the expectations of consumers, beef producers must ensure the production of beef of consistently high quality. Ultimate meat tenderness is controlled by inherent complex mechanisms, which include an interaction of muscle biochemistry with muscle processing, which in turn are influenced by a number of factors including breed (Cuvelier *et al.,* 2006; Gagaoua *et al.*, 2019), age at slaughter (Nian *et al*., 2017) and type of muscle (Picard and Gagaoua, 2020). In addition, genetics and management, which control gene expression (Hocquette *et al*., 2007), as well as variables associated with *post-mortem* meat processing (Short *et al.,* 1999), could also affect meat quality. Epigenetics, is another factor that regulates gene expression and potentially

affects production characteristics and meat quality. According to Satheesha et al. (2021), epigenetics is the study of the changes in gene expression that are heritable, as well as other genomic functions without changes to the underlying DNA sequence. Epigenetics is therefore associated with the expression of genes and the expression of phenotypes. The expression of genes due to epigenetics is primarily because of the effects of the internal and external environment surrounding an organism. The changes associated with epigenetics contribute to adaptation on a short-term basis of individuals, which can be reversed (Satheesha *et al*., 2021). Thus, epigenetics characterizes the mechanisms that explain changes in phenotype rather than changes in the sequence of DNA. Additionally, epigenetic changes regulate the expression of genes and are dependent on external factors including environment and nutrition. As an example, studies on protein nutrition have been carried out to enhance productive traits and have aimed at determining the extensive protein, as well as essential amino acids requirements in pigs. This ultimately influences the growth rate of the pigs, and the quality of their final product (Marín-García and Llobat, 2021).

Regulatory mechanisms that underlie the differences observed in meat quality remain largely unknown (te Pas *et al.,* 2010). Gene expression controls the biological characteristics of muscles (Sami *et al.,* 2015). The knowledge of genes and their expression levels would allow a better understanding of the physiological processes that occur in muscles, and their influence on meat quality (Hocquette *et al.,* 2007). The quantification of gene expression has become a necessity in most molecular biology laboratories. This is due to its high sensitivity, large dynamic range and little or no post-amplification processing. It is also accommodating to increasing sample throughput (Wong and Medrano, 2005).

Differential expression of genes (mRNA abundance) or proteins could explain the differences in meat quality. This may be used to develop biomarkers in the prediction and monitoring of meat quality (Wimmers *et al.,* 2007; te Pas *et al.,* 2009). Genes of the calpain family, μ-calpain (*CAPN1*), m-calpain (*CAPN2*), and calpain 3, are commonly studied candidate genes due to their contribution to the degradation of myofibrillar protein and *post-mortem* proteolysis (Ono *et al.,* 2016). Calpastatin (CAST), which is the inhibitor of the calcium-dependent proteases (CAPN1 and CANP2), is also involved in the viability and proliferation of cells (Fang *et al.,* 2016). These proteins of the calpain family (CAPN1, CAPN2 and CAST) are associated with meat

tenderness in different cattle populations that are well documented (Lian *et al.*, 2013). Aside from the *CAST* and *CAPN* genes, other genes associated with beef tenderness have been found (Marty *et al.*, 2010). Cattle with mutations in the myostatin gene were found to produce meat of improved tenderness (Wheeler *et al*., 2001; Lines *et al.*, 2009). Additionally, an association was discovered in the Piemontese cattle breed between a polymorphism in intron 3 of the *growth hormone 1* gene and beef tenderness at 11 days *post-mortem* (Di Stasio *et al.,* 2003). The gene *DNAJA1* has also been found to be associated with meat tenderness through gene expression analysis (Bernard *et al.*, 2007). In the study by Bernard et al. (2007), the gene *DNAJA1* alone explained 63% of the variation in beef tenderness, observed in *longissimus thoracis* muscles from 25 Charolais bull calves.

To increase the understanding of the pathways and processes that contribute to variation in beef tenderness, several studies have been conducted using omics tools (Gagaoua *et al.*, 2019). Additionally, omics tools have been used to propose prediction equations that explain the variability observed (Gagaoua *et al.*, 2020). The application of analytical technologies and bioinformatics tools related to omics in recent decades has resulted in a greater understanding of gene expression, physiological responses, and other metabolic processes that are involved in the determination of meat quality, and more specifically tenderness (Picard and Gagaoua, 2017; Picard and Gagaoua, 2020). An evolving group of disciplines, foodomics include transcriptomics and proteomics, which is applied to food and characteristics related to food quality. Foodomics has been extensively used to study fresh meat, as well as meat products (Munekata *et al.*, 2021). Proteomics in particular has played a key role in the discovery of target biomarkers of a number of meat quality characteristics (Gagaoua *et al.*, 2021). In comparison with traditional methods used in the evaluation of beef tenderness with the aid of instruments or sensory methods, an enhanced procedure for monitoring beef quality using rapid methods to measure the abundance of specific proteins involved would provide a benefit of predicting the quality of meat before it is consumed (Zhu *et al.,* 2021). Furthermore, these methods can potentially be enhanced to predict future tenderness phenotype throughout the production period, to slaughter and consumption (Gagaoua *et al.*, 2017; Picard and Gagaoua, 2017).

The extent to which a particular gene is expressed can be determined based upon the measure of cellular RNA. Different experimental conditions can cause the expression of genes to change

significantly (Pike *et al.,* 2005). Genes can also vary in their expression levels depending on the cell in which they are found. The study of quantitative gene expression can be used to validate the extent of transcription of a gene, as well as protein levels (Pike *et al.,* 2005). Real time (RT)-PCR remains a robust tool used to quantify gene expression where accurate quantification of protein abundance is not possible. Gene expression is therefore a useful but under-utilized tool in the elucidation of the impacts of animal production practices on proteins that directly control the synthesis and degradation of collagen. With the advancement of molecular genetics, several genes involved in the production of meat have been identified. However, our understanding of the biochemical and physiological mechanisms underlying the operation of these genes involved in meat production remain limited. Furthermore, although several studies have focused on the expression of several genes and their contribution to meat tenderness in the muscle fibre and connective tissue, studies that focus on treatment effects such as growth promotant use, on gene expression levels in relation to meat quality, particularly meat tenderness are deficient. Studies in this area are warranted given that most cattle production systems in North America employ the use of growth promotants. Results from this study will add to knowledge regarding the synthesis of collagen in meat animal muscle so that meat of high quality, especially of consistent desired tenderness, can be produced.

4.2 Objective

This study used gene expression analysis to identify the effect of growth promotant treatment and RFI status on proteins associated with collagen and collagen crosslink synthesis, and to understand their relationships with collagen and meat quality characteristics, particularly toughness as estimated using Warner-Bratzler shear force.

This was achieved by testing the following hypotheses:

1. Steers of low RFI status are expected to have lower expression levels of genes associated with collagen synthesis including *SMAD2, SMAD3, FN1, ITGA1, ITGA11* and *ITGB1,* higher expression levels of genes associated with collagen crosslink synthesis including *LH1, LH3* and *LOX*, low expression of genes involved in protein degradation including *MMPs* and *TIMPs*, and have higher gene expression levels of calpastatin (*CAST*)

2. Genes associated with collagen and collagen crosslinks synthesis are highly expressed in muscles with high shear force values.

3. Growth promotants increase the expression levels of genes of proteins responsible for collagen and collagen crosslinks, and hence increase the background toughness of meat.

4.3 Materials and Methods

Muscle samples from the *semimembranosus* (SM) muscles harvested from 47 crossbred Angus steers were used in this study. The management and euthanasia of the experimental animals have been described in detail in Chapter 2 of this thesis. Measurement of carcass and meat quality traits, proximate analysis, and blood metabolite analyses have also been described previously in Chapter 2 of this thesis, and measurement of the characteristics of collagen heat-solubility, and collagen crosslink concentrations have been described in detail in Chapter 3 of this thesis. Data from these analyses and originally presented in these chapters were related statistically to gene expression data described in this chapter.

4.3.1 RNA Source, Extraction, Quantity and Quality

For gene expression analysis, *semimembranosus* muscle samples were obtained post-exsanguination, frozen in liquid nitrogen and transported on dry ice to the laboratory where they were stored at -80 °C until analyzed. For extraction of RNA, a muscle piece was removed without thawing and was ground into powder with sterilized mortar and pestle using liquid nitrogen. About 0.1 g of the ground samples was measured into bead tubes and stored at -80 °C until ready to use. One (1) mL of TRIzol® was added to the ground muscle sample in each bead tube and samples homogenized in a Precellys 24 homogenizer (Bertin Technologies, SA, Montigny-le-Bretonneux, France). RNA was then precipitated using isopropanol (Fisher Scientific, Ottawa, Ontario) and 1.2 M sodium acetate in 0.8 NaCl. Precipitated RNA pellet was washed with 75% ethanol. The RNA was finally solubilized in 50 µL of molecular grade water.

4.3.2 Evaluation of Isolated RNA

The concentration, purity and quality of extracted RNA was evaluated based on absorbance at 260 nm and 230 nm, using an ND-1000 spectrophotometer (Nanodrop Technologies, USA). RNA Integrity Number (RIN) was determined with an Agilent Tapestation (2200 Bioanalyzer,

Agilent Tapestation, UK). RNA integrity was deemed adequate if a number greater than 7 was obtained. The range of RNA Integrity Number (RIN) obtained in this analysis was between 7.4 and 8.4.

4.3.3 Reverse transcription of RNA (Complementary DNA (cDNA) synthesis

cDNA was synthesised from the original RNA samples (1 µg) using the iScript™ Reverse Transcription Supermix (Bio-Rad, USA) for RT-qPCR and RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, $MgCl_2$, and stabilizers (Bio-Rad, USA). A negative control (NO-RT) was prepared with iScript NO-RT Control Supermix (Bio-Rad, USA) and contained all components of the iScript RT Supermix with the exception of the reverse transcriptase. This was used to serve as a no-enzyme control to ensure cDNA samples were uncontaminated with genomic DNA. The reaction mix was incubated in a thermal cycler (Applied Systems, USA) following the protocol form the manufacturer. This consisted of a priming step for 5 minutes at 25 ℃, a reverse transcription step for 20 minutes at 46 ℃, and finally, an RT inactivation step for 1 minute at 95 ℃. The cDNA was then diluted with nuclease-free water and stored at -20 ℃ until used for gene expression analysis.

4.3.4 Primer Design for candidate or target genes

Forward and reverse primers for 27 of the 31 genes identified to be associated with the synthesis and degradation of intramuscular connective tissue (IMCT) and collagen were designed in a previous study from our laboratory group (Hamed, 2020 (Thesis)) using Primer Express Software (AB Applied Biosystems, USA; Annex 1). The coding sequence for the forward and reverse primers of these genes were obtained from the NCBI Blast database. For the primer express software (AB Applied Biosystems, USA, Version 3.0.1) used, the default setting included 20-24 base primer length and 20-30 base probe length. The %GC was 30-80 while the amplicon size was set at 50-150 with primer melting temperature set between 58-60 ℃. The sequence for the forward and reverse primers of the remaining target genes were obtained from literature and verified using UCSC In-Silico PCR. The primer sequences are listed below in Table 4.1. The genes used in this study were *TGFB1, FGF2, ITGB1, LOX, MMP2, MMP8, MMP9, MMP13, FGFR1, P4HA1, TIMP1, TIMP2, SMAD2, SMAD3, SMAD4, SMAD6, SMAD7, IGF-1, LH1, LH3, COL1A1, COL3A1, COL5A1,COL6A1, CAST, CAPN1, ITGA1, ITGA11, DNAJA1, PITX2* and *FN1* (Table 4.2).

Table 4.1 Forward and reverse primers for target genes.

Gene name	GenBank accession	Sequence (5'- 3')	Tm ℃	Annealing ℃	Amplicon size bp
TGFB1	NM_001166068.1	Forward CTGACCCGCAGAGAGGAAATA Reverse GGTTCATGCCGTGAATGGTG	59.2 59.8	60	142
FGF2	NM_174056.4	Forward CCACTTCAAGGACCCCAAGC Reverse GTAGTTTGATGTGTGGGTCGC	60.9 59.5	60	129
ITGB1	NM_174368.3	Forward GCCTTGCATTGCTGCTGATT Reverse CAGTTGTCACAGCACTCTTG	60.1 57.0	60	138
LOX	NM_173932.4	Forward ACACACACAGGGCTTGAGTC Reverse TCAGGCACCAAATAGCTGGG	60.2 60.0	60	138
MMP9	NM_174744.2	Forward CCATTAGCACGCACGACATC Reverse GAGGTCGAAGGTCACGTAGC	59.7 60.2	60	131
FGFR1	NM_001110207.1	Forward GGCAGTGACACCACCTACTT Reverse AGCCACGGGGTTTGGTTTG	59.6 61.1	60	129
P4HA1	NM_001075770.1	Forward GGACTGTTTTGAGTTGGGCAAA Reverse CGGTAGAAACCTCGCCTTCA	59.8 59.8	60	110
TIMP1	NM_174471.3	Forward GATGTCGTCATCAGGGCCAA Reverse GGGTGTAGATGAACCGGATG	60.1 57.8	60	145
TIMP2	NM_174472.4	Forward TCTGGCAACGACATCTACGG Reverse TTCCTCCAATGTCCAGCGAG	59.8 59.8	60	151
MMP2	NM_174745.2	Forward TGATGGCGCCCATTTATACC Reverse GCCGGTGCCAGTATCAATGT	58.1 60.8	60	110
MMP8	XM_024975688.1	Forward TTTCCTGTTGCTGCCCATGA Reverse ATGCAGTGAGTAGCTGCTGG	60.2 60.1	60	117
SMAD2	NM_001046218.1	Forward GGAACTGCCCCCTCTGGAT	61.0	60	110

		Reverse ATCCAGGAGGTGGCGTTTCT	61.2		
SMAD3	NM_001205805.1	Forward TGAAGCGAAGTTTGGGCGG Reverse GCAGGATGGACGACATGGTT	61.3 61.4	60	136
SMAD4	NM_001076209.1	Forward CCCCATCCCGGACATTACT Reverse CGATCTCCTCCAGAAGGGTCTA	58.5 60.2	60	200
SMAD6	NM_001206145.1	Forward CCTGGGACCTGAGACAGAGTTG Reverse CTTCCTTCTTACTCCCTGCAAAAA	61.99 59.17	60	130
SMAD7	NM_001192865.1	Forward GGCATTCCTCGGAAGTCAAGA Reverse CATCTGGACAGTCTGCTGTGGATT	60.1 60.3	60	189
IGF-1	NM_001077828.1	Forward CCATCTCCCTGGATTTCTTTTTG Reverse GAAGAGATGCGAGGAGGATGTG	57.7 60.6	60	177
LH1	NM_174148.1	Forward TCCACTACCCCCAAAAACGG Reverse GGCATCCACGCTGAAGTAGT	59.9 60.1	60	218
COL1A1	NM_001034039.2	Forward CGAGGAAATGATGGTGCGAC Reverse CTTCACCCTTAGCACCCACAG	59.4 60.6	60	100
COL3A1	NM_001076831.1	Forward ATGTTGTGCAGTTTGCCCAC Reverse AGGACCAGGATCGCCATTTC	59.9 59.8	60	127
COL5A1	XM_024999726.1	Forward CGCCTCCCACAGTGTAACG Reverse GCCTCAATTCAGTTCTTGCAAA	60.0 60.1	60	98
COL6A1	NM_001143865.2	Forward CGACTGCGCCATCAAGAAG Reverse CCGTCAGTCACCACAACCAA	59.3 60.5	60	90
CAST		Forward GTGCCCAGGA CCCCA TTG Reverse AGCAGGCTTCTTGTCTTTGTC	64.8 58.7	60	1464
CAPN1		Forward CATGGTCAACCTCATGGAT Reverse TTAGGGTCACCTGTAGACGAG	56.6 56.9	60	244
MMP13	NM_174389.2	Forward TTGTTGGTCTCTGCCCCTTC	59.9	60	148

		Reverse AATCACAGAGCTTGCTGCAG	58.8		
ITGA1	XM_005221521.4	Forward CACCAACCCAAAAGGAGGGT Reverse TGGGGCTGACATCAGAACAG	60.1 60.0	60	101
ITGA11	XM_002690525.6	Forward GCCTACAGCACCGTCCTAAA Reverse TCGATGCTGCCATCTGAGTC	59.8 59.9	60	89
DNAJA1		Forward AGGGTCGCCTAATCATTGAA Reverse TCCTCGTATGCTTCTCCATTG	59.5 60.2	60	199
LH3		Forward ATGAGGATACGCAGGGTCTG Reverse CTGGGCCTGGGAGAGAGGAGTG	60.1 65.9	60	
PITX2	NM_001097991	Forward CCGAAGACCCGTCCAAGAA Reverse GCTGCATAAGCCCGTTGAAC	59.3 60.2	60	270
FN1	NM_001163778.1	Forward TCAGAGACGGGCAAGAGAGA Reverse AGTAATGTCGGGAGTGGTGC	60.0 59.8	60	146

Table 4.2 Functions of target genes for collagen synthesis and degradation.

Gene	Gene name	Gene product functions	References
FN1, FGFR1, FGF2	Fibronectin, fibroblast growth factors	Signalling proteins, involved in cell proliferation and development, and wound healing. Regulate fibrogenic proliferation and differentiation	Mohammadi et al. (2005) Seung (2018)
COL1A1,COL3A1, COL5A1, COL6A1	Collagen genes for collagen types I, III, V and VI Alpha chains	Regulation of collagen synthesis, provide strength and support to growing muscle	Gonzalez et al. (2014) Ricard-Blum and Ruggiero (2005)
LOX	Lysyl oxidase	Initiation and regulation of collagen crosslink formation	Kagan and Li, 2006
LH1, LH3	Lysyl hydroxylase 1, lysyl hydroxylase 3	Also known as procollagen-lysine, 2-oxoglutarate 5-dioxygenase (*PLOD1, PLOD3*). Regulation of collagen crosslink synthesis through hydroxylation of lysine residues found in helical domains of fibrillar and non-fibrillar collagens	Valtavaara et al. (1998) Wang et al. (2007)
IGF-1, TGF-β1	Insulin-like growth factor 1, transforming growth factor - *β1*	Implicated in stimulation of collagen synthesis	Nam et al. (1997)

SMAD2,SMAD3,SMAD4,SMAD6, SMAD7	SMAD related proteins	Transduction of signals to nucleus, through downstream actions from serine/threonine kinase receptors of the *TGF-β* family. SMADs 2,3,4,6 regulate collagen expression in a steady state SMADs 6 and 7 inhibit the actions of SMAD related proteins	Massagué and Wotton (2000)
MMP2, MMP8, MMP9, MMP13	Matrix metalloproteinases	Involved in collagen degradation	Overall (2004)
TIMP1, TIMP2	Tissue inhibitor of matrix metalloproteinases	Regulate activities of extracellular active enzymes, directed at a range of components of the extracellular matrix. Inhibitors of MMPs	Morrison et al. (2009)
P4HA1	Collagen prolyl 4-hydroxylase	Involved in collagen biosynthesis, controls the formation of collagen triple helix	Myllyharju, (2003)
PITX2	Paired-like homeodomain transcription factor 2	Important for the growth and proliferation of cells in different tissues. Regulates the activities of procollagen enzymes such as lysyl hydroxylase	Ghosh (2007) Shah et al. (1997)
CAST	Calpastatin	Inhibitor of calpain enzyme	Dunner et al. (2013)

CAPN1	μ-calpain	Involved in *post-mortem* muscle proteolysis	Dunner et al. (2013)
ITGA1, ITGA11, ITGB1	Integrins	Binds to ligands including collagen types I, III, IV IX XIII, and XVI	Gardner (2014)
DNAJA1	DnaJ Heat Shock Protein Family (Hsp40) Member	Anti-apoptotic protein, encodes the heat-shock protein 40 (Hsp40). Negative selection marker for meat tenderness	Kayani et al. (2008) Gotoh et al. (2011)

4.3.5 Quantitative Polymerase Chain Reaction (qPCR) for the testing and selection of housekeeping (HK) genes and new target genes

Six (6) HK genes, namely glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), B-Actin (*ACTB*), 18S ribosomal RNA, hydroxymethylbilane synthase (*HMBS*), splicing factor 3 subunit 1 (*SF3A1*) and eukaryotic translation elongation factor 1 alpha 2 (*EEFIA2*), were considered. Three (3) cDNA samples were used to test all six HK genes, and the new target genes, *LH3*, *DNAJA1, CAST* and *CAPN1*. For the amplification of the HK and target genes, a master mix for each gene was prepared consisting of 1 µL of forward primer, 1 µL of reverse primer and 10 µL of SYBR® green (Applied Biosystems). Axygen® PCR microplates (Corning Life Sciences, USA) were used for this test. A single reaction consisted of 7 µL of nuclease-free water, 12 µL of mastermix and 1 µL of diluted cDNA samples.

Primer specificity was further confirmed with the melting curve after amplification. Primers that produced unspecified products (had double or more peaks or a product in the negative (no template control) did not go through further testing and were not used in this study. The plate was run in a 96 well plate reader (Thermofisher, Applied Biosystems, USA). The programme used in the thermocycler (Thermofisher, Applied Biosystems, USA) was preheating at 95 °C for 20 seconds, denaturation at 95 °C for 3 seconds, annealing and elongation at 60 °C for 30 seconds, melt curve stage at 95 °C for 15 seconds, 60 °C for 1 minute, and 95 °C for 15 seconds. One of the HK genes, *EEFIA2,* failed to be expressed when tested with the three cDNA samples and was therefore not further used in the study. Following testing of the HK, their consistency was further tested in all 47 cDNA samples.

4.3.6 Selection of housekeeping (HK) or reference genes

Expression levels of the HK genes tested were determined by cq values. A standard curve was obtained and the equation for estimating the quantity of gene was:

Quantity of gene = Efficiency $^{(\text{minCt} - \text{Ct sample})}$, where Efficiency = $10^{\wedge(-1/\text{slope})}$

and minC_t is the minimum C_t value of the housekeeping gene

The cq values were used to determine three HK genes that were not influenced by treatment. The C_t value range from amplification plots, and peaks shown on melt curves were used as an additional combined criterion for the selection of the two most appropriate reference or HK genes. The best

two HK genes were chosen based on the cq value (without influence from the treatments used in the study), consistent C_t values (narrow C_t range) and single peak (production).

4.3.7 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) for the expression of target genes

For cDNA from both target and reference genes, three (3) technical replicates were used to generate an average expression to ensure technical errors were minimal. Original cDNA samples were diluted 40 times (2 µL of cDNA sample + 78 µL nuclease-free water) for this purpose and used as templates. Mastermix for each primer was prepared by adding 2 µL nuclease-free water, 0.5 µL each of forward and reverse primers and 5 µL of SYBR® green (Applied Biosystem). Each reaction mix contained 2 µL diluted cDNA and 8 µL mastermix for the HK and target genes in a MicroAmp fast optical 348 well plate (Applied Biosystem, Singapore). The complete reactions were run in a ViiA7 (Applied Biosystems, USA) with an initial holding step for heating and activating polymerase at 95 °C for 20 seconds, a cycling/denaturation step at 95 °C for 3 seconds, and an annealing step at 60 °C for 30 seconds. The number of cycles for amplification was set at 40.

4.3.8 Statistical analyses

As two (2) HK genes were used, the average of their C_t values was obtained. The relative expression levels for each of the 31 target genes were calculated by the mean differences of their C_t values and the average C_t values of the HK genes to obtain their ΔC_t values. The mean ΔC_t value is inversely proportional to the number of amplicons; therefore, the higher the mean ΔC_t value, the lower the level of amplification, and vice versa (Livak *et al.*, 2001). The general linear model (GLM) procedure of R Software (Version 3.5.1) was used to fit an appropriate model. Expression level data of the 31 target genes were analysed using a 2 x 2 x 2 factorial with RFI status, steroid and ractopamine hydrochloride and their interactions as the fixed effects. The least squares mean of each treatment was compared using Tukey's Honestly Significant Difference (HSD) test. Differences were considered significant at $P \leq 0.05$. Pearson's correlation coefficient analysis was performed to identify linear relationships between the expression levels of the target genes and phenotypic measurement of growth (growth performance traits), carcass and meat quality characteristics, and collagen heat-solubility characteristics, using the rcorr function in the

Harrel Miscellaneous Package (Hmisc) package in R Studio. A Bonferroni correction was applied by dividing the alpha value of 0.05 by the number of variables (Rice, 1989).

4.4 Results

4.4.1 Housekeeping (HK)/reference gene selection

Five (5) HK genes were tested using PCR. *18S* is a housekeeping gene that has been tested and used in our laboratory for gene expression experiments, and has proven to be a consistent housekeeping gene for lyophilized meat samples (Ijiwade, 2019 (Thesis); Hamed, 2020 (Thesis)). *SF3A1* and *HMBS* are new considerations to the regular housekeeping genes (*18S, ACTB* and *GAPDH*) used in our laboratory as they have been reported in literature to be more stable compared to the conventional housekeeping genes (*18S, ACTB* and *GAPDH*). Two (2) of the HK genes tested, *18S* and splicing factor 3 subunit 1 *SF3A1*, were selected as the most stable reference genes for the muscle samples as detected by qPCR. The main rationale behind the selection of these two HK genes was that they were unaffected by the treatments used in this study (RFI status, Steroid, RH and their interactions) (Table 4.3).

Table 4.3 Effect of RFI, steroid and Ractopamine hydrochloride (RH) on mean expression level (cq) (with standard error in parentheses) of housekeeping genes.

Housekeeping genes	RFI		Steroid		Ractopamine hydrochloride		P Value		
	Low	High	Steroid	Control	RH	Control	RFI	Steroid	RH
ATCB	0.284 (0.04)	0.324 (0.04)	0.369 (0.04)[a]	0.239 (0.04)[b]	0.295 (0.04)	0.313 (0.04)	0.5078	0.0343	0.7607
GAPDH	0.315 (0.05)	0.348 (0.05)	0.416 (0.05)[a]	0.247 (0.05)[b]	0.339 (0.05)	0.323 (0.05)	0.6596	0.0275	0.8308
HMBS	0.087 (0.05)	0.090 (0.05)	0.086 (0.04)	0.091 (0.04)	0.056 (0.04)	0.120 (0.04)	0.9684	0.9379	0.2754
18S	0.292 (0.05)	0.292 (0.05)	0.361 (0.05)	0.223 (0.05)	0.318 (0.05)	0.266 (0.05)	0.9950	0.0646	0.4722
SF3A1	0.369 (0.05)	0.450 (0.06)	0.379 (0.05)	0.440 (0.06)	0.446 (0.05)	0.374 (0.05)	0.3051	0.4367	0.3596

[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$

[1] Probability of the F test, with significance at $P \leq 0.05$

In addition to *18S* and *SF3A1* being affected neither by the treatments nor their interactions, they were chosen over *HMBS* because *HMBS* had a wider range of C_t values (19-33) as seen on the amplification plot (Figures 4.1, 4.2 and 4.3) among samples compared to *18S* (13-21) and *SF3A1* (26-30). Furthermore, all samples tested with *18S* and *SF3A1* showed specified products (single peaks), while samples tested with HMBS showed some unspecified products (double peaks) (Figures 4.4, 4.5 and 4.6). Thus, considering all three selection parameters (cq value (based on treatment effect), C_t value range from amplification plots, and peaks from the melt curve plots), *18S* and *SF3A1* were better housekeeping genes than *HMBS*.

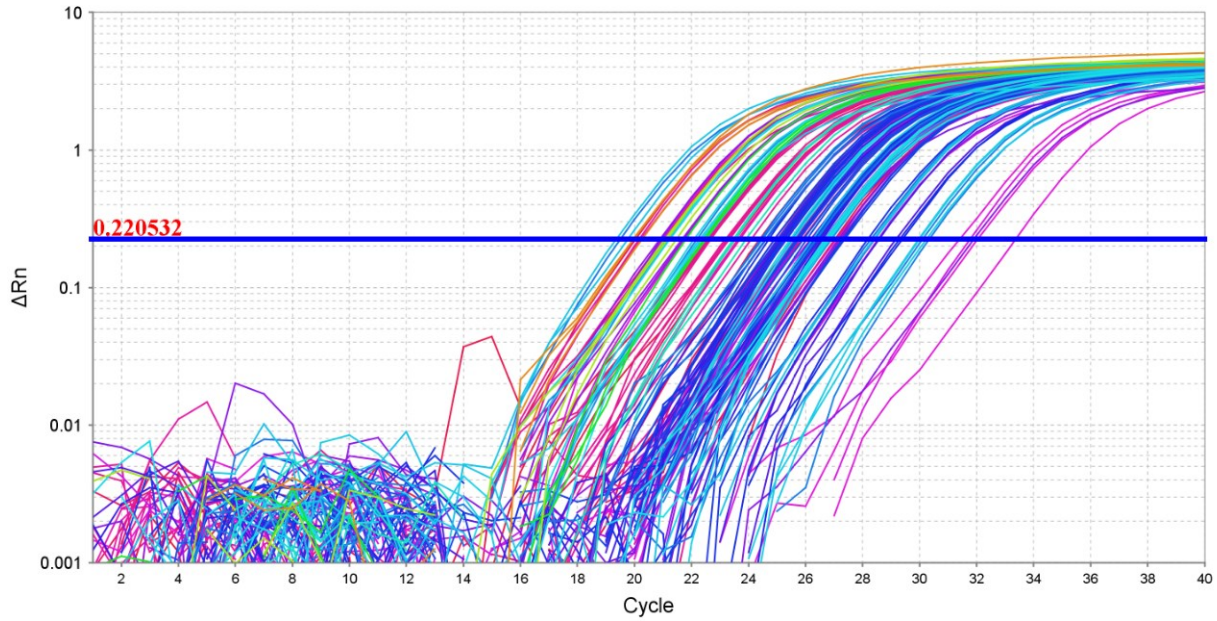

Figure 4.1 Amplification plot showing C_t range for the housekeeping gene HMBS. C_t range: 19-33.

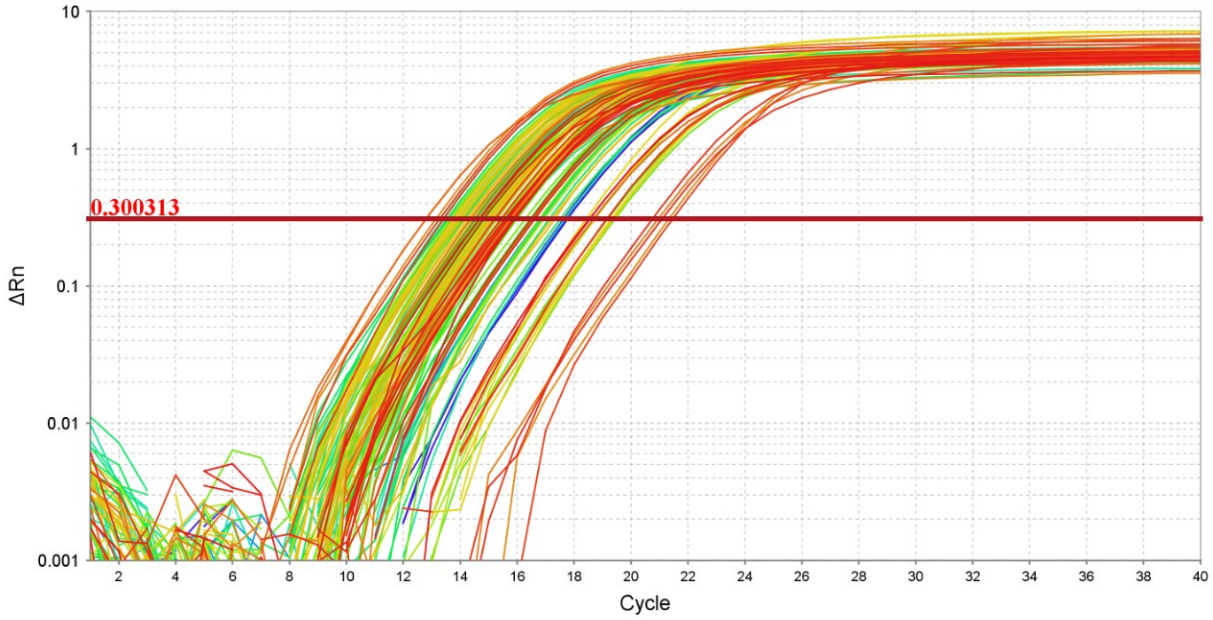

Figure 4.2 Amplification plot showing C_t range for the housekeeping gene 18S. C_t range:13-21

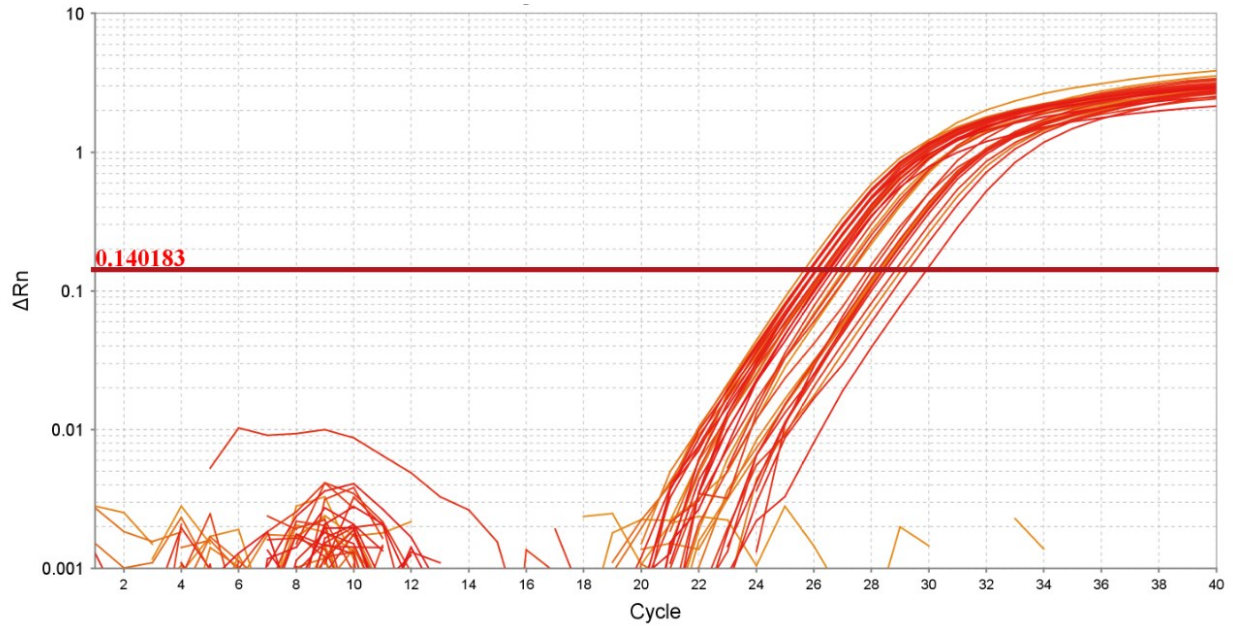

Figure 4.3 Amplification plot showing C_t range for the housekeeping gene SF3A1. C_t range: 25-30.

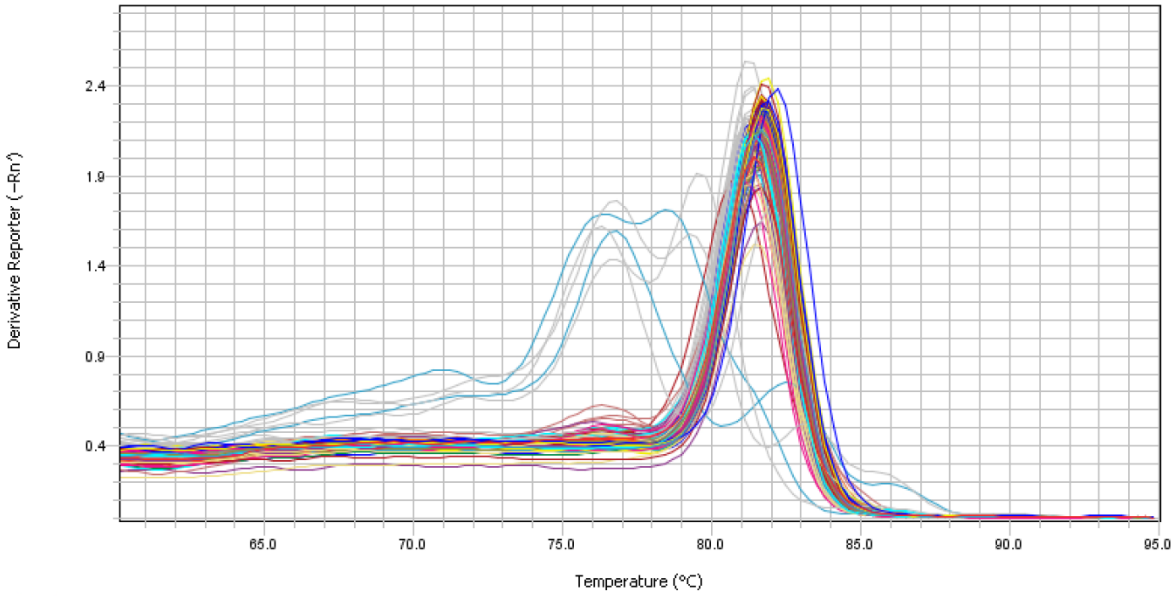

Figure 4.4 Melt curve plot for the housekeeping gene *HMBS. HMBS* shows unspecified products (double peaks) for some samples.

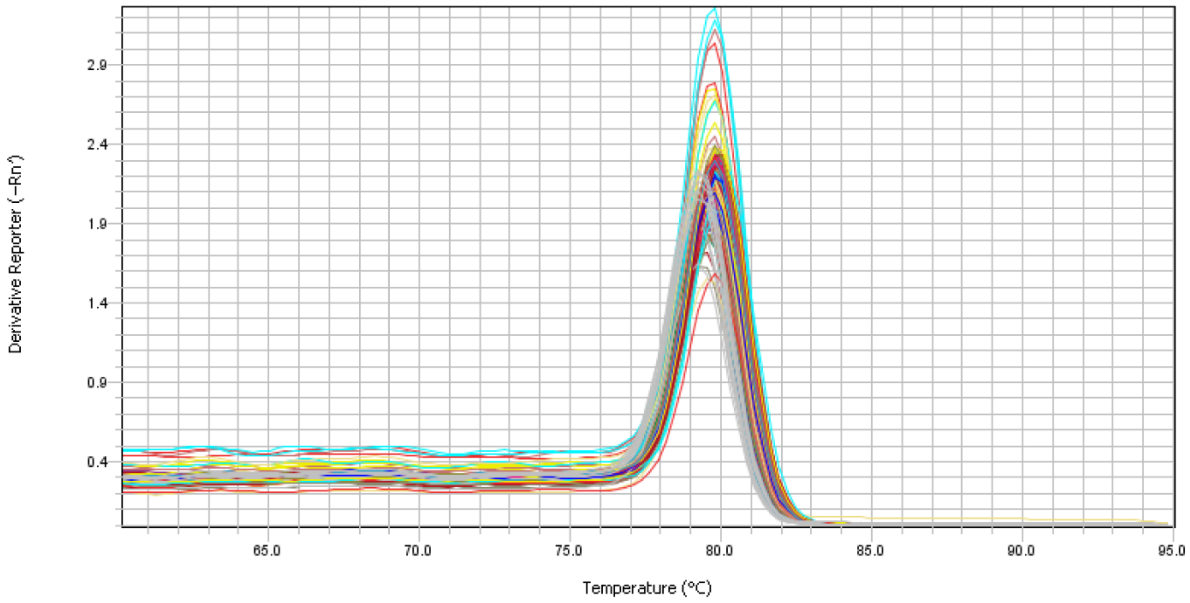

Figure 4.5 Melt curve plot for the housekeeping gene *18S. 18S* shows specified products (single peaks) with all samples tested.

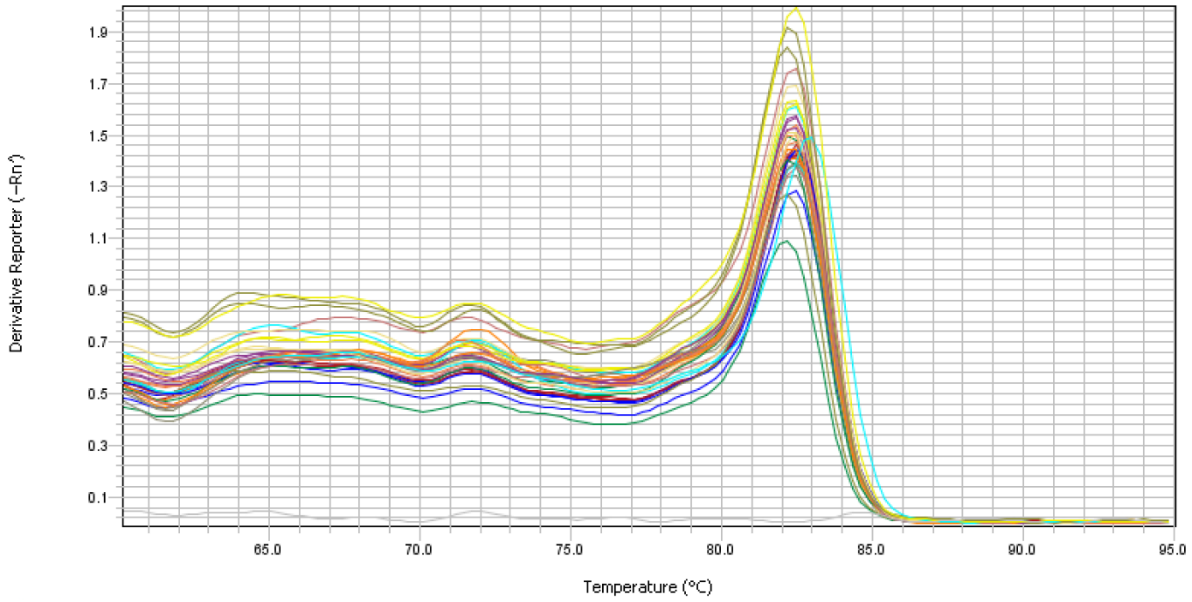

Figure 4.6 Melt curve plot for the housekeeping gene *SF3A1*. *SF3A1* shows specified products (single peaks) with all samples tested.

4.4.2 Effect of treatments and their interaction on target genes

A three-way interaction between RFI, steroid and RH on the expression of the *CAPN1* gene was observed (Figure 4.7). The results showed the highest expression of the *CAPN1* gene occurred in the muscle of steers of high RFI that were both implanted and supplemented with RH, while steers of low RFI that were also implanted and RH supplemented had the lowest expression of the *CAPN1* gene ($P = 0.0168$) (Figure 4.7). Although the interaction effect was statistically significant, the means of the different treatment combinations were not significantly different. The interaction appeared to be driven by growth promotants, where low RFI steers treated with either steroids or RH tended to have higher expression of *CAPN1* and lower expression when treated with both compared to high RFI steers (Figure 4.7).

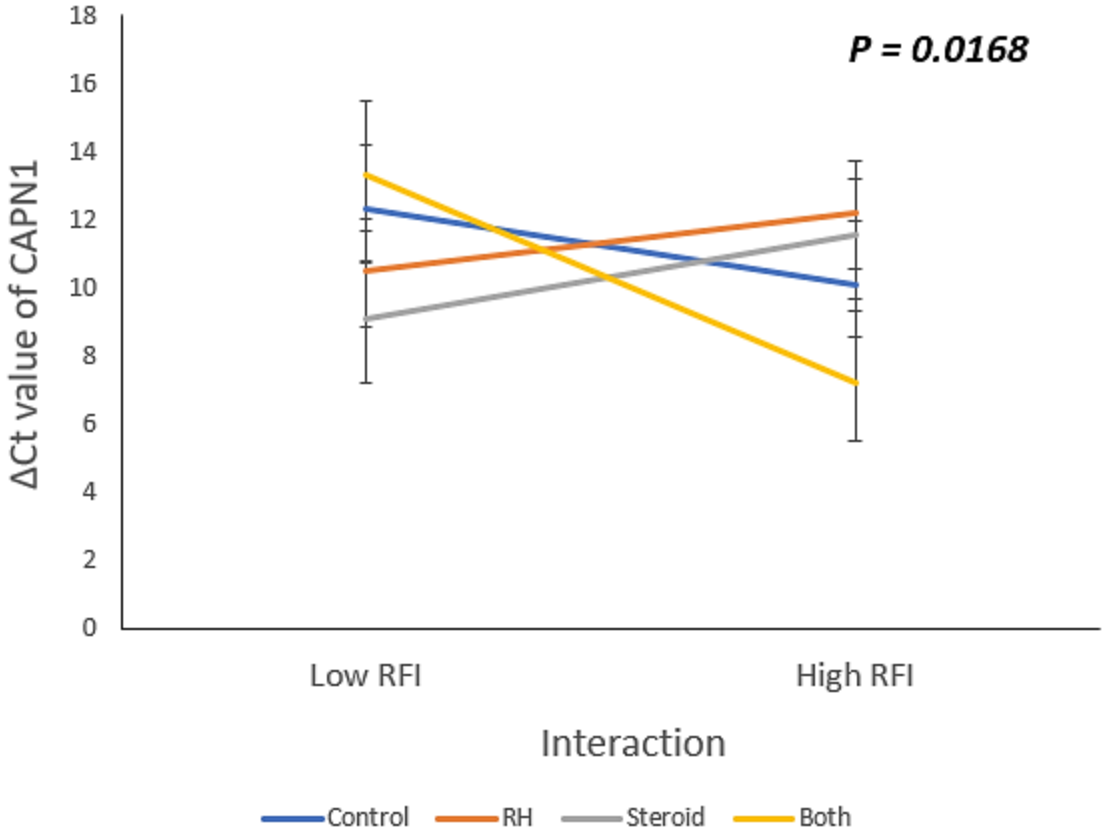

Figure 4.7 Gene expression level of *CAPN1* in the SM muscles from steers selected for RFI, and subjected to steroid implantation or not and RH supplementation or not. (Muscles from steers of Low RFI status, implanted and not RH supplemented (n = 6); Muscles from steers of low RFI status, non-implanted and RH supplemented (n = 7); Muscles from steers of low RFI status, non-implanted and not RH supplemented (n = 6); Muscles from steers of low RFI status, implanted and RH supplemented (n = 7); Muscles from steers of high RFI status, implanted and RH supplemented (n = 5); Muscles from steers of high RFI status, non-implanted and not RH supplemented (n = 6); Muscles from steers of high RFI status, implanted and not RH supplemented (n = 5); Muscles from steers of high RFI status, non-implanted and RH supplemented (n = 5).

Results from analysis of variance showed that selection for low (efficient) and high (control) RFI status did not influence the expression levels (mean ΔC_t values) of the target genes tested (Table 4.4). The effect of RFI status on the expression of *SMAD7* however approached significance (P = 0.0910), where muscle samples from efficient (low RFI) steers tended to have lower expression of *SMAD7* compared to muscle from control steers (Table 4.4). The effect of RFI on *CAST* expression level also approached significance, where muscle from low RFI steers tended to have higher *CAST* expression compared to muscles from high RFI steers (P = 0.0903). Likewise,

MMP13 tended to be expressed at a lower level in muscles from low RFI steers compared to muscles from high RFI steers ($P = 0.0859$) (Table 4.4).

Table 4.4 Effect of RFI on gene expression level (ΔC_t) of target genes (means with standard errors presented).

	RFI		
Target genes	Low	High	P Value[1]
TGFB1	9.20 (0.15)	8.96 (0.16)	0.2628
FGF2	5.71 (0.38)	5.02 (0.42)	0.2289
ITGB1	2.66 (0.17)	2.67 (0.20)	0.9670
LOX	5.75 (0.23)	5.60 (0.25)	0.6761
MMP9	7.28 (0.56)	6.99 (0.64)	0.7374
FGFR1	8.61 (0.27)	8.41 (0.29)	0.6202
P4HA1	6.18 (0.25)	6.24 (0.27)	0.8883
TIMP1	6.83 (0.23)	7.01 (0.25)	0.5933
TIMP2	2.82 (0.17)	3.12 (0.18))	0.2364
MMP2	5.58 (0.20)	5.72 (0.22)	0.6463
MMP8	6.70 (0.73)	6.61 (0.79)	0.9364
SMAD2	1.33 (0.49)	1.64 (0.54)	0.6744
SMAD3	10.00 (0.30)	9.46 (0.33)	0.2337
SMAD4	5.13 (0.43)	4.96 (0.46)	0.7960
SMAD6	8.89 (0.27)	8.38 (0.30))	0.2102
SMAD7	11.80 (0.27)	11.10 (0.30)	0.0910
IGF-1	8.99 (0.17)	8.97 (0.18)	0.9310
LH1	7.85 (0.25)	7.69 (0.26)	0.6541
COL1A1	7.27 (0.17)	7.22 (0.18)	0.8351

COL3A1	6.63 (0.23)	6.59 (0.27)	0.9216
COL5A1	8.04 (0.26)	7.76 (0.28)	0.4791
COL6A1	4.77 (0.19)	4.71 (0.21)	0.8382
CAST	6.68 (0.78)	8.58 (0.83)	0.0903
CAPN1	11.30 (0.91)	10.20 (0.84)	0.3908*
MMP13	9.93 (0.45)	8.78 (0.48)	0.0859
ITGA1	5.27 (0.19)	5.26 (0.21)	0.9645
ITGA11	9.51 (0.16)	9.33 (0.17)	0.4777
DNAJA1	1.47 (0.35)	1.41 (0.38)	0.9020
LH3	10.80 (0.30)	10.50 (0.33)	0.4096
PITX2	12.4 (0.33)	12.50 (0.36)	0.8556
FN1	4.19 (0.15)	3.96 (4.19)	0.3093

[1] Probability of the F test, with significance at $P \leq 0.05$

* Gene is involved in an interaction significant at $P \leq 0.05$.

The results from this study showed a significant effect of steroid implantation on the mean ΔC_t values of *ITGB1*, *TIMP2, CAST* and *ITGA11* ($P < 0.05$) (Table 4.5). For the gene *ITGB1*, mean ΔC_t values revealed a lower expression level in muscle samples from implanted steers compared with muscle samples from non-implanted steers ($P = 0.0125$) (Table 4.5). The expression level for *TIMP2* was higher in muscle samples from non-implanted steers than in muscle samples from implanted steers ($P = 0.0445$). *CAST* expression level in samples from non-implanted steers was higher compared to that of implanted steers ($P = 0.0405$). For *ITGA11*, the mean ΔC_t values showed higher expression in muscle samples from non-implanted steers than in muscle samples from implanted steers ($P = 0.0221$). The results also showed a steroid effect on *FN1,* which approached significance ($P = 0.0752$), where a tendency for greater *FN1* expression level was observed in muscle samples from non-implanted steers than in muscle samples from implanted steers (Table 4.5).

Table 4.5 Effect of steroids on gene expression level (ΔC_t) of target genes (means with standard errors presented).

Target genes	Steroid		*P* Value[1]
	Steroid	Control	
TGFB1	9.13 (0.16)	9.02 (0.15)	0.6070
FGF2	5.58 (0.42)	5.15 (0.38)	0.4489
ITGB1	3.01 (0.20)[a]	2.32 (0.17)[b]	0.0125
LOX	5.92 (0.25)	5.44 (0.23)	0.1698
MMP9	6.77 (0.64)	7.51 (0.56)	0.3892
FGFR1	8.57 (0.29)	8.45 (0.27)	0.7704
P4HA1	6.39 (0.27)	6.03 (0.25)	0.3367
TIMP1	7.09 (0.25)	6.75 (0.23)	0.3166
TIMP2	3.23 (0.18)[a]	2.71 (0.17)[b]	0.0445
MMP2	5.80 (0.22)	5.49 (0.20)	0.2948
MMP8	6.98 (0.79)	6.33 (0.73)	0.5542
SMAD2	1.98 (0.54)	0.99 (0.49)	0.1834
SMAD3	9.66 (0.33)	9.79 (0.30)	0.7735
SMAD4	4.83 (0.47)	5.26 (0.42)	0.4951
SMAD6	8.61 (0.30)	8.65 (0.27)	0.9283
SMAD7	11.20 (0.30)	11.70 (0.27)	0.2034
IGF-1	9.06 (0.18)	8.90 (0.17)	0.5206
LH1	8.03 (0.26)	7.51 (0.25)	0.1547
COL1A1	7.42 (0.18)	7.07 (0.17)	0.1704
COL3A1	6.69 (0.27)	6.53 (0.25)	0.6552

COL5A1	7.85 (0.28)	7.95 (0.26)	0.7915
COL6A1	4.87 (0.21)	4.62 (0.19)	0.3756
CAST	8.79 (0.84)[a]	6.47 (0.77)[b]	0.0405
CAPN1	10.30 (0.92)	11.30 (0.82)	0.4243*
MMP13	9.24 (0.46)	9.47 (0.47)	0.7869
ITGA1	5.36 (0.20)	5.16 (0.20)	0.4768
ITGA11	9.71 (0.18)[a]	9.13 (0.16)[b]	0.0221
DNAJA1	1.78 (0.38)	1.10 (0.35)	0.1983
LH3	10.40 (0.33)	10.90 (0.30)	0.2520
PITX2	12.20 (0.35)	12.70 (0.34)	0.2626
FN1	4.29 (0.17)	3.87 (0.15)	0.0752

[1] Probability of the F test, with significance at $P \leq 0.05$

[a, b] Least square means within a row lacking a common letter differ at $P \leq 0.05$

* Target gene is involved in an interaction significant at $P \leq 0.05$.

Supplementation of crossbred Angus steers with ractopamine hydrochloride (RH) did not affect the expression levels of any targeted genes ($P > 0.05$) (Table 4.6). Furthermore, no tendencies ($P \leq 0.1$) were observed.

Table 4.6 Effect of RH on gene expression level (ΔC_t) of target genes (means with standard errors presented).

Target genes	RH		*P* Value[1]
	RH	Control	
TGFB1	9.20 (0.16)	8.96 (8.64)	0.6070
FGF2	5.41 (0.40)	5.32 (0.40)	0.8833
ITGB1	2.87 (0.19)	2.45 (0.183)	0.1170
LOX	5.82 (0.24)	5.53 (0.24)	0.3981
MMP9	6.73 (0.61)	7.54 (7.54)	0.3464
FGFR1	8.70 (0.29)	8.32 (0.28)	0.3470
P4HA1	6.11 (0.26)	6.31 (0.26)	0.5982
TIMP1	6.75 (0.24)	7.10 (0.24)	0.3054
TIMP2	3.05 (0.18)	2.89 (0.17)	0.5070
MMP2	5.72 (0.21)	5.57 (0.21)	0.6139
MMP8	6.00 (0.76)	7.31 (0.76)	0.2343
SMAD2	1.25 (0.52)	1.75 (0.51)	0.5173
SMAD3	9.61 (0.32)	9.85 (0.32)	0.5896
SMAD4	5.32 (0.45)	4.77 (0.44)	0.3886
SMAD6	8.61 (0.27)	8.65 (0.28)	0.9255
SMAD7	11.40 (0.29)	11.40 (0.28)	0.9039
IGF-1	9.04 (0.18)	8.91 (0.17)	0.5929
LH1	7.93 (0.26)	7.61 (0.25)	0.3865
COL1A1	7.29 (0.17)	7.20 (0.17)	0.6947
COL3A1	6.43 (0.26)	6.79 (0.26)	0.3399
COL5A1	7.74 (0.27)	8.06 (0.27)	0.4042

COL6A1	4.59 (0.20)	4.89 (0.20)	0.2931
CAST	7.63 (0.79)	7.63 (0.81)	0.9961
CAPN1	10.8 (0.88)	10.8 (0.87)	0.9707*
MMP13	9.24 (0.46)	9.47 (0.47)	0.7307
ITGA1	5.36 (0.20)	5.16 (0.20)	0.4756
ITGA11	9.52 (0.17)	9.32 (0.17)	0.4156
DNAJA1	1.59 (0.37)	1.29 (0.37)	0.5670
LH3	10.50 (0.31)	10.80 (0.31)	0.4688
PITX2	12.70 (0.35)	12.20 (0.34)	0.2869
FN1	4.21 (0.16)	3.95 (0.16)	0.2615

[1] Probability of the F test, with significance at $P \leq 0.05$

* Target gene is involved in an interaction significant at $P < 0.05$.

4.4.3 Pearson's correlation coefficients

Results showed a moderate positive correlation between *FGF2* and *TGF-β1* ($P < 0.001$). *ITGB1* was moderately positively correlated with *TGF-β1* and *FGF2* ($P < 0.001$) (Table 4.7). The gene *LOX* was highly positively correlated with *FGF2* and *ITGB1* ($P < 0.001$). *FGFR1* had moderate positive correlations with *TGF-β1* and *FGF2* ($P < 0.001$). *TIMP1* was moderately positively correlated with FGF2 and highly positively correlated with *P4HA1* ($P < 0.0001$). The gene *MMP2* had moderate positive correlations with *ITGB1* and *P4HA1*, and a high positive correlation with *TIMP1* ($P < 0.001$). *MMP2* had near-significant moderate positive correlations with *TGF-β1* ($P = 0.0044$), *FGF2* ($P = 0.0021$), *FGFR1* ($P = 0.0024$) and *TIMP2* ($P = 0.0015$). *MMP8* was moderately positively correlated with *TGF-β1*, *FGF2*, *P4HA1* and *TIMP1* ($P < 0.001$) and a low positive correlation with *TIMP2* ($P = 0.0014$) (Table 4.7). A strong positive correlation existed between *MMP2* and *TIMP1* ($P < 0.0001$). *SMAD2* was moderately positively correlated with *TGF-β1*, *FGF2*, *ITGB1* and *FGFR1*, and highly positively correlated with *P4HA1* and *TIMP1* ($P < 0.0001$). *SMAD3* had moderate positive correlations with *TGF-β1* and *FGF2* ($P < 0.001$) (Table 4.7). The gene *SMAD6* had moderate positive correlations with FGF2 and *LOX* ($P < 0.001$). *SMAD7* was moderately positively correlated with *TGF-β1* ($P = 0.0011$) and *FGF2* ($P < 0.001$) (Table 4.7).

Table 4.7 Pearson's correlation coefficients between target genes of the *semimembranosus* (SM) muscle.

	TGFB1	*FGF2*	*ITGB1*	*LOX*	*MMP9*	*FGFR1*	*P4HA1*	*TIMP1*	*TIMP2*	*MMP2*	*MMP8*	*SMAD2*	*SMAD3*	*SMAD4*	*SMAD6*
FGF2	**0.47***														
ITGB1	**0.48***	**0.56***													
LOX	0.25	**0.65***	**0.68***												
MMP9	0.1	-0.12	-0.24	-0.05											
FGFR1	**0.60***	**0.49***	0.24	0.16	-0.18										
P4HA1	0.45	0.44	0.31	0.19	0.09	0.23									
TIMP1	0.34	**0.49***	0.27	0.13	-0.03	0.3	**0.77***								
TIMP2	0.12	-0.02	0.28	-0.01	-0.02	0.03	0.28	0.36							
MMP2	0.41	0.44	**0.51***	0.34	-0.31	0.44	**0.60***	**0.71***	0.45						
MMP8	**0.48***	**0.52***	0.06	0.03	0.24	0.41	**0.68***	**0.69***	0.10	0.29					
SMAD2	**0.50***	**0.67***	**0.48***	0.24	-0.06	**0.45***	**0.77***	**0.85***	**0.41***	0.72	0.72				
SMAD3	**0.51***	**0.62***	0.15	0.28	0.25	0.4	0.25	0.28	-0.14	0.12	0.54	0.41			
SMAD4	-0.09	-0.34	-0.28	-0.38	0.26	-0.12	-0.04	-0.14	-0.1	-0.29	-0.15	0.08	0.08		
SMAD6	0.18	**0.48***	0.39	**0.57***	0.05	-0.12	0.24	0	-0.38	0.03	0.17	0.25	0.25	-0.17	
SMAD7	**0.46***	**0.49***	0.37	0.41	0.07	0.32	0.1	0	-0.06	0.09	0.24	0.49	0.49	-0.23	0.4

*Asterisks beside each correlation indicate probability with significance at $P < 0.001$. An r value ≥ an absolute value of 0.29 is significant at $P = 0.05$.

The results showed moderate positive correlations between *TGF-β1* and *LH1*, and *DNAJA1* ($P <$ 0.001) (Table 4.8). *FGF2* was highly positively correlated with LH1 and *DNAJA1*, moderately positively correlated with *COL5A1*, MMP13, *ITGA1*, LH3 and *FN1* ($P < 0.001$). *FGF2* was moderately negatively correlated with *COL3A1* ($P < 0.001$). A moderate negative correlation also existed between *ITGB1* and *COL3A1* ($P < 0.001$), while a moderate positive correlation was found between *MMP9* and *CAST* ($P = 0.0015$), a moderate negative correlation was found between *FGFR1* and *CAST* ($P = 0.0012$). *LOX* had moderate positive correlations with *LH1* and *FN1* ($P <$ 0.001) (Table 4.7). *FGFR1* had a high positive correlation with *ITGA1*, and had moderate positive correlations with *ITGA11*, *DNAJA1*, and *FN1* ($P <0.001$) (Table 4.8). The results also revealed a moderate positive correlation between *TIMP1* and *COL5A1* ($P = 0.0011$) (Table 4.8). A moderate positive correlation was found between *TIMP2* and *COL6A1* ($P < 0.001$). The gene *MMP8* had a moderate negative correlation with *IGF-1* ($P < 0.001$). *MMP8* was moderately positively correlated with *DNAJA1* ($P = 0.0011$). SMAD2 was moderately positively correlated with *LH1*, *ITGA1*, and *DNAJA1* ($P < 0.001$). *SMAD2* was also moderately positively correlated with *COL5A1* (P = 0.0018) and *FN1* (0.0013). Moderate positive correlations were found between *SMAD3* and *LH1*, and *LH3* ($P < 0.001$) (Table 4.8). A moderate positive correlation was found between *SMAD6* and *LH1*, and *MMP13* ($P <0.001$). *SMAD6* was also moderately negatively correlated with *COL3A1* $P < 0.001$). The gene *SMAD7* was moderately positively correlated with *LH1* ($P < 0.001$), and with *LH3* ($P = 0.0014$) (Table 4.8).

Table 4.8 Pearson's correlation coefficients between target genes of the *semimembranosus* (SM) muscle.

	IGF-1	*LH1*	*COL5A1*	*COL6A1*	*CAST*	*CAPN11*	*MMP13*	*COL1A1*	*ITGA1*	*ITGA11*	*DNAJA1*	*LH3*	*PITX2*	*COL3A1*	*FN1*
TGFβ1	-0.15	**0.57***	0.17	0.02	-0.02	-0.18	0.12	0.2	0.42	0.37	**0.47***	0.07	-0.12	-0.12	0.45
FGF2	-0.2	**0.71***	**0.54***	-0.05	-0.04	0.35	**0.56***	0.31	**0.65***	0.26	**0.75***	**0.58***	-0.28	**-0.64***	**0.68***
ITGB1	0.31	**0.69***	0.23	0.22	0.22	-0.01	0.29	0.07	**0.51***	0.26	**0.66***	0.17	-0.25	**-0.46***	**0.69***
LOX	0.35	**0.64***	0.28	0.01	0.27	0.13	0.39	0.27	**0.36***	**0.34***	**0.45***	**0.45***	-0.19	-0.44	**0.60***
MMP9	0.28	0.2	-0.25	-0.12	**0.46***	-0.27	-0.03	0.15	-0.37	0.08	-0.28	0.09	0.3	0.21	-0.24
FGFR1	-0.4	0.29	0.39	0.14	**-0.47***	0.08	0.09	0.32	**0.72***	**0.49***	**0.58***	0.1	-0.02	-0.11	**0.47***
P4HA1	-0.32	0.34	0.32	-0.03	0.08	0.03	0.33	0.13	0.17	0.08	0.36	0.1	-0.12	-0.07	0.31
TIMP1	-0.32	0.29	**0.46***	0.15	-0.11	-0.01	0.34	0.09	0.26	-0.02	0.33	0.12	-0.02	-0.05	0.25
TIMP2	-0.1	0.04	0.18	**0.54***	0.14	-0.1	-0.06	-0.07	0.07	0.13	0.17	-0.21	-0.16	0.2	0.04
MMP2	-0.15	0.29	0.39	0.27	-0.16	0.02	0.21	0	0.43	0.07	0.36	-0.01	-0.28	-0.16	0.42
MMP8	**-0.52***	0.4	0.37	-0.13	0.01	0.05	0.29	0.33	0.31	0.23	**0.46***	0.26	-0.001	0.04	0.24
ITGB1	-0.34	**0.57***	**0.44***	0.12	0.02	0.07	0.32	0.16	**0.53***	0.16	**0.64***	0.25	-0.26	**-0.30***	**0.46***
LOX	-0.17	**0.56***	0.43	-0.06	0.06	-0.11	0.4	0.34	0.39	0.15	0.43	**0.58***	0.16	-0.33	0.35
MMP9	0.29	-0.15	-0.12	-0.09	-0.05	-0.31	-0.09	-0.11	-0.23	-0.04	-0.26	-0.1	0.19	0.06	-0.26
FGFR1	0.18	**0.49***	0.17	-0.41	0.3	0.28	**0.50***	0.07	0.04	0.03	0.23	0.39	-0.31	**-0.52***	0.41
P4HA1	0.04	**0.51***	0.21	0	0.02	0.08	0.27	0.12	0.39	0.17	0.43	**0.45***	-0.11	-0.38	0.39
TIMP1	-0.15	**0.57***	0.17	0.02	-0.02	-0.18	0.12	0.2	0.42	0.37	**0.47***	0.07	-0.12	-0.12	0.45
TIMP2	-0.2	**0.71***	**0.54***	-0.05	-0.04	0.35	**0.56***	0.31	**0.65***	0.26	**0.75***	**0.58***	-0.28	**-0.64***	**0.68***
SMAD2	0.31	**0.69***	0.23	0.22	0.22	-0.01	0.29	0.07	**0.51***	0.26	**0.66***	0.17	-0.25	**-0.46***	**0.69***
SMAD3	0.35	**0.64***	0.28	0.01	0.27	0.13	0.39	0.27	**0.36***	**0.34***	**0.45***	**0.45***	-0.19	-0.44	**0.60***
SMAD4	0.28	0.2	-0.25	-0.12	**0.46***	-0.27	-0.03	0.15	-0.37	0.08	-0.28	0.09	0.3	0.21	-0.24
SMAD6	-0.4	0.29	0.39	0.14	**-0.47***	0.08	0.09	0.32	**0.72***	**0.49***	**0.58***	0.1	-0.02	-0.11	**0.47***
SMAD7	-0.32	0.34	0.32	-0.03	0.08	0.03	0.33	0.13	0.17	0.08	0.36	0.1	-0.12	-0.07	0.31

*Asterisks beside each correlation indicate probability with *$P < 0.001$. An r value ≥ an absolute value of 0.29 is significant at $P = 0.05$.

The genes *COL5A1* and *COL6A1* were moderately positively correlated (P = 0.0002) (Table 4.9). *MMP13* had a moderate positive correlation with *COL5A1* (P < 0.0001). A moderate positive correlation existed between *ITGA1* and *LH1* (P < 0.0001). Moderate and positive correlations were found between *LH3* and *LH1* (P < 0.0001), and *LH3* and *MMP13* (P = 0.0002) (Table 4.9). *ITGA11* had moderate positive correlations with *COL1A1* (P = 0.0002), and with *ITGA1* (P = 0.0014). Moderate positive correlations existed between *DNAJA1* and *LH1* (P < 0.0001), and *DNAJA1* and *COL5A1* (P = 0.0008). *DNAJA1* was strongly positively correlated with *ITGA1* (P < 0.001). The gene *COL3A1* had moderate negative correlations with *LH1*, *MMP13*, *DNAJA1* and *LH3* (P < 0.001), and *ITGA1* (P = 0.0012). While moderate positive correlation existed between *FN1* and *LH1*, *ITGA1*, *ITGA11* and *DNAJA1*, a moderate negative correlation existed between *FN1* and *COL31A1* (P < 0.001) (Table 4.9).

The gene *FGFR1* and initial weight before steroid treatment were moderately negatively correlated (r = -0.60) (P < 0.0001). Similarly, a moderate negative correlation existed between *MMP8* and initial weight before steroid (r = -0.54) (P < 0.0001). A moderate positive correlation existed between *SMAD2* and feed conversion efficiency during the RH supplementation period (r = 0.52) (P = 0.0002). The gene *COL5A1* was moderately negatively correlated with initial weight before steroid treatment (r = -0.49) (P = 0.0005). A positive moderate relationship was found between the *CAST* gene, and initial steer weight before steroid treatment (r = -0.40) (P = 0.0054), while a low positive correlation was found between CAST and final steer weight after second steroid application (r = 0.38) (P = 0.0085), all of which approached significance. At day 3 *post-mortem,* the target genes were not significantly correlated with the meat quality characteristics of the SM muscle (P > 0.001). At day 12 *post-mortem*, a moderate negative correlation existed between pH and *FGFR1* (r = -0.56) (P < 0.0001). The gene *P4HA1*, and cook loss were moderately positively correlated (r = 0.46) (P < 0.001) at day 12 *post-mortem*. At day 3 *post-mortem*, total collagen content of the SM muscle was moderately negatively correlated with *SMAD6* (r = -0.46) (P = 0.0012). Genes were not significantly correlated with collagen heat-solubility measures at day 12 *post-mortem*.

Table 4.9 Pearson's correlations between target genes of the *semimembranosus* (SM) muscle.

	IGF-1	*LH1*	*COL5A1*	*COL6A1*	*CAST*	*CAPN11*	*MMP13*	*COL1A1*	*ITGA1*	*ITGA11*	*DNAJA1*	*LH3*	*PITX2*	*COL3A1*
LH1	0.17													
COL5A1	-0.33	0.22												
COL6A1	0.04	-0.05	**0.51***											
CAST	-0.22	0.21	-0.34	-0.17										
CAPN11	0.21	0.12	0.04	0.84	-0.3									
MMP13	-0.16	0.4	**0.60***	0.19	-0.04	0.27								
COL1A1	0.01	0.28	0.07	-0.18	0.23	0.04	-0.13							
ITGA1	-0.24	**0.57***	0.41	0.14	-0.25	0.23	0.21	**0.35***						
ITGA11	0.11	0.32	0.02	-0.04	0.06	0.11	-0.19	**0.53***	**0.46***					
DNAJA1	-0.25	**0.66***	**0.47***	0.11	-0.06	0.33	0.31	0.29	**0.80***	0.42				
LH3	0.01	**0.59***	0.33	-0.13	0.1	0.2	**0.52***	0.29	0.47	0.03	0.42			
PITX2	-0.02	0.06	0.01	0.06	0	-0.19	0.23	0.11	-0.06	-0.07	-0.18	0.35		
COL3A1	-0.03	**-0.61***	-0.27	0.1	-0.06	-0.34	**-0.53***	0.09	**-0.46***	0.2	**-0.47***	**-0.53***	0.05	
FN1	0.04	**0.59***	0.33	-0.05	-0.02	0.3	0.31	0.23	**0.61***	**0.47***	**0.69***	0.32	-0.17	**-0.48***

*Asterisks beside each correlation indicate probability with *$P < 0.001$. An r value ≥ an absolute value of 0.29 is significant at $P = 0.05$

4.5 Discussion

Tenderness is one of the principal factors that influence beef acceptability. It is also the palatability trait of cooked meat that is widely studied (Beermann, 2009). Meat tenderness is influenced by multiple factors such as genetics, nutrition, growth promotants, chilling and *post-mortem* ageing, and this makes it a subject of continuous study (Tatum *et al.,* 2007). Tenderness can vary as a function of growth rate, and in response to differences in the concentrations of collagen types I and III as well (Subramaniyan and Hwang, 2017). Also, turnover and concentration of collagen may vary among muscles in the same animal, most likely due to their position and function in the body, resulting in variations in meat tenderness among different muscles (Archile-Contreras *et al.,* 2010). As an example, postural muscles such as the *longissimus thoracis et lumborum* muscle contains a minimal concentration of collagen and collagen trivalent crosslinks and is therefore a more tender cut compared to a muscle such as the *semimembranosus* (SM), which is involved in locomotion and contains a higher concentration of collagen and collagen trivalent crosslinks (Dubost *et al.,* 2013). According to Young and Gregory (2001), the toughening of meat is related to the increase in mature cross-links that are non-reducible, which has been observed with increase in animal age. The mature crosslink pyridinoline (PYR) is considered to be chiefly involved in this process (Lawrie and Ledward, 2006).

In the current study, we investigated the effect of selection for RFI and growth promotants on the level of expression of SM muscle genes associated with proteins involved in collagen and collagen crosslink synthesis. Overall, the expression levels of genes that encode 31 proteins implicated in meat toughness and collagen synthesis and degradation were profiled in 47 SM muscle samples. This is the first study that has explored the effect of growth promotants and selection for RFI on the expression levels of genes associated with these proteins. Results from this study showed an interaction between RFI status, steroid, and RH supplementation on the expression level of *CAPN1* in the SM muscle. The calpain proteolytic system has been identified as one of the factors responsible for the process of meat tenderization *post-mortem.* The *CAPN1* gene and its inhibitor calpastatin encoded by *CAST* (Koohmaraie, 1996) together affect the Warner-Bratzler Shear Force (WBSF) of muscle (Leal-Gutiérrez, 2018). The interaction showed that steers of low RFI status, implanted and RH supplemented had the lowest expression level of the *CAPN1* gene. Several reports from different studies have shown that the proteolysis of major

myofibrillar proteins by *CAPN1* is the fundamental mechanism of *post-mortem* tenderization of meat that occurs during storage at refrigerated temperatures (Ouali and Talmant, 1990; Koohmaraie *et al.,* 1995). According to Oddy (1999), the turnover of protein in living animals involves high amounts of energy. Low RFI steers are associated with lower protein turnover rates, relative to high RFI steers (Oddy, 1999). Genetic selection for growth has been shown to be associated with variation in protein metabolism (Oddy, 1999). Selection for RFI, particularly low RFI may therefore produce changes in the calpain system. This may be as a result of the efficient use of energy, leading to a lower protein turnover rate in such animals, which ultimately impacts meat tenderness negatively (McDonagh *et al.*, 2001). Thus, *CAPN1* level would be expected to be reduced in low RFI steers, however, the interaction observed for *CAPN1* appeared driven by treatment with growth promotants, as similar expression levels of *CAPN1* were observed between control low and high RFI steers. Low RFI steers, treated with hormonal steroids and supplemented with RH had lower expression of *CAPN1*. This indicates that low and high RFI steers responded differently to growth promotants. Theoretically, growth promotants favour reduced protein degradation during muscle hypertrophy. Given that *CAPN1* degrades protein, the use of hormonal implants and supplementation with RH would be expected to result in the reduction of its expression level.

Interestingly, where steroids and RH when used individually they appeared to have no effect on mRNA expression of the *CAPNI* gene expression. Bearing in mind the different mechanisms through which hormonal implants and RH result in muscle hypertrophy (detailed in Chapter 1 of this thesis), one would expect additive effects of the two growth promotants on the mRNA expression of the *CAPN1* gene, as was observed in the current study. Further studies are warranted on the effects of the two growth promotants on gene expression levels to ascertain if they are indeed additive or not. Further in this thesis, the expression of *CAPN1* was neither correlated with WBSF nor with any of the heat-solubility characteristics and collagen crosslinks, or the *CAST* gene, suggesting that a balance between the expression levels of the two genes is not directly responsible for the difference in WBSF values of the SM muscle due to steroid treatment in particular.

Aside from *CAPN1*, RFI status did not substantially affect any of the 31 target genes profiled, particularly the expression levels of genes of encoding proteins associated with collagen

synthesis and degradation. The study by Chen et al. (2011) was the first to explore global gene expression profiling using the bovine microarray to identify genes that are differentially expressed in relation to feed efficiency in beef cattle. A total of 161 unique genes that were differentially expressed were identified using 22 high and 22 low RFI bulls. In the said study by Chen et al. (2011), *COL1A1* and *COL3A1* were upregulated in low RFI Angus cattle through interaction with platelet-derived growth factor, which does not agree with results found in the current study, where similar expression levels of *COL1A1, COL3A1* and all genes implicated in collagen synthesis and degradation were obtained for low and high RFI steers. *COL1A1* and *COL3A1* are associated with the activity of the ECM and upregulation of these genes in steers of low RFI status indicates higher activity in the ECM of efficient steers compared to less efficient steers (Chen *et al.,* 2011). In the human adult, high expression of genes of the ECM including *COL1A1* and *COL3A1* is usually an indication of a fibrotic liver (Bedossa and Paradis, 2003). In highly efficient (low RFI) animals however these same genes are potentially related to physiological functions that are normal for growth and include cell proliferation, migration, differentiation, and ultimately gene expression (Chen *et al.,* 2011). *COL1A1* and *COL3A1* are known to be associated with collagen synthesis. Other genes implicated in collagen synthesis include *COL5A1, COL6A1, SMAD2, SMAD3,* and FN1 and expression levels of these genes were similar for low and high RFI steer muscles, which is an indication of similar collagen synthesis rate in low and high RFI steers, which may mean similar intramuscular collagen content, as was reported in Chapter 3 in the GM and SM muscles. *TIMP1* and *TIMP2* were also upregulated in low RFI steers in the study by Chen et al. (2011), and that was not observed in this thesis. It was hypothesized that due to differences in energy metabolism, particularly with respect to protein turnover, collagen synthesis genes in low RFI steers would be downregulated. This hypothesis was rejected as similar levels of gene expression of associated proteins involved in collagen and collagen crosslink synthesis were obtained for low and high RFI steers. The difference between the results in the present study and the study by Chen et al. (2011) could be due to the different breeds of steers used. While purebred Angus steers were used in the study by Chen et al. (2011), crossbred Angus steers were used in the present study.

The amount of collagen in the skeletal muscle is controlled by the balance between collagen synthesis and degradation (Zhao *et al.*, 2015). MMPs are important for the degradation of the ECM including collagen (Verna and Hansch, 2007). TIMPs are proteins that specifically inhibit the activity of the MMPs. A balance between MMPs and TIMPs is therefore essential for the proper

remodeling of the ECM (Gupta *et al.*, 2014). MMP13*,* known to be involved in connective tissue turnover, tended to have a lower expression in low RFI animals. In normal cells, the expression level of *MMP13* is low, which contributes to a healthy remodeling of the connective tissue (Vincenti and Brinckerhoff, 2002). Collagen types I, II and III are the main targets of the collagenase MMP13. The tendency for *MMP13* to have reduced expression in low RFI steers is an indication that there may be reduced connective tissue remodeling in muscles from low RFI steers, which was expected. When the turnover of collagen, especially of types I and III collagen because they are known to be associated with meat toughness due to their abundance in skeletal muscles (Light *et al*., 1985) is reduced, it may result in the production of meat that is tough. Results of WBSF reported in Chapter 2 of this thesis support the tendency of *MMP13* to be downregulated in muscles from low RFI steers, as SM muscles from low RFI steers were tougher than those from high RFI steers. RFI status did not result in differential expression of *TIMPs* in the current study. Similar expression levels of *TIMP* in muscles regardless of RFI status indicated equal opportunity for *MMP13* activity to be inhibited in muscles from both low and high RFI steers. However, with *MMP13* being differentially expressed in muscles from low and high RFI steers, there is the potential for the contribution of collagen to beef toughness to be magnified in low RFI steers with intensive selection for this trait.

The effect of RFI status on the expression of the *CAST* gene in the *semimembranosus* (SM) muscle approached significance and indicated that steers of low RFI status had the tendency to highly express the *CAST* gene in the SM muscle. This tendency for SM from low RFI steers to have increased *CAST* expression was associated with an increased mean WBSF value relative to muscles from high RFI steers as reported in Chapter 2 of this thesis. This agrees with a study by McDonagh et al. (2001) where crossbred steers selected for low RFI had 13% more calpastatin level compared to crossbred steers selected for high RFI in the *longissimus thoracis et lumborum* muscle. In the study by McDonagh et al. (2001), the ratio of μ-calpain to calpastatin had a positive correlation with the rate of myofibrillar fragmentation, which was similar to the results of McDonagh et al. (1999) for lamb, and in electrically stimulated beef (McDonagh *et al.,* 1998). McDonagh et al. (2001) found that the higher the calpastatin level, the lower the rate of myofibrillar fragmentation as a result of calpain inhibition in the *longissimus thoracis et lumborum* muscle. Selection for low RFI may therefore also mean selection for increased expression of *CAST* and less tender meat (McDonagh *et al.,* 2001).

Results from the current study also showed the tendency for *SMAD7* to be expressed at a lower level in SM muscles from low RFI steers. Within the family of R-SMADS, SMAD3 mediates the production of collagen in fibroblasts and is stimulated by TGF-β (Chin *et al.,* 2001). SMAD7 is an inhibitor SMAD that regulates negative feedback and can antagonize the activity of R-SMADs. This is achieved through the binding of SMAD7 to the phosphorylated type I receptor of TGF-β, which results in the obstruction of the TGF-β pathways (Dienus *et al.*, 2010), and subsequently results in reduced collagen synthesis. Low RFI steers in the current study was reported in the second thesis chapter to have a higher body weight at the end of the combined steroid implant phase compared to high RFI steers. This increase in the body weight of low RFI steers is possibly due to an increase in muscle mass due to a reduction in protein degradation that may have resulted from increased *CAST* expression. The increase in muscle mass demands an increase in connective tissue synthesis to support it. As a result, one would expect *SMAD7* to be downregulated in muscles from low RFI steers, as was realized in the current study, where *SMAD7* had the potential to be downregulated in muscles from high RFI steers. Further studies on the regulation of gene expression, particularly of the family of SMADs by RFI status are therefore warranted.

Steroids had the greatest effect on the expression levels of the 31 target genes. Steroid application decreased the expression of *ITGA11, ITGB1, TIMP2* and *CAST* in this thesis. The use of hormonal implants has been associated with increased lean deposition, hence increased protein synthesis and retention (Johnson *et al.*, 1996; Kerth *et al.* 2003; Dunshea *et al.* 2005; Duckett and Pratt 2014). It was therefore expected that collagen synthesis as estimated by mRNA expression of genes associated with proteins involved in collagen synthesis would also increase to enable the connective tissue to accommodate the growing muscle. The action of hormones shows that the regulation of gene expression is not limited to alterations in gene transcription rate because they can stabilize a particular mRNA to modify its concentration in a steady state (Ing, 2005).

Results from the current study showed that *ITGB1* and *ITGA11* were downregulated in the SM muscle by steroid application, contrary to the upregulation of genes associated with collagen and collagen synthesis such as *MMP,* and an increase in the transcription of collagen molecules reported in the study by Gonella-Diaza et al. (2018). *ITGB1* and *ITGA11* are part of a family of integrins, and bind to collagen molecules (Zeltz *et al.,* 2016). According to Heino et al. (2000), the

intracellular domains of integrins could be connected to tyrosine-kinase and adapter proteins, which further link them to cellular signaling pathways, including that involved in collagen synthesis. Activation of MMP1 and MMP13 was found to be dependent on signals produced after the integrin ITGA1 interacted with collagens (Riikonen *et al*., 1995, Rivanti *et al*., 1999). The expression of integrins *ITGB1* and *ITGA11* has been shown to contribute to the binding of collagen type I and the production of collagen (Gardner *et al.,* 1996; Gardner, 1999). Growth factors are the main regulators of the expression of integrins at the tissue level. In humans, TGF-β is the growth factor that controls the transcription of many integrins in various cell types and tissues, and mostly stimulates the expression of integrin (Pechkovsky *et al.,* 2008). ITGA11 is a chief receptor of fibrillar collagen in fibroblasts. It pairs with β1 integrins, and together binds to collagen I with high affinity (Tiger *et al.,* 2001; Popova *et al*., 2007), and is involved in the differentiation of fibroblasts and reorganization of collagen (Carracedo *et al*., 2010). According to Talior-Volodarsky et al. (2014), *ITGA11* transcription is regulated by TGF-β1 through the SMAD2/3 transcription factors which binds with the *ITGA11* proximal promoter (Lu *et al.*, 2006), or by TGF-β2 through the binding action of SMAD2/3 to a sequence in the *ITGA11* distal promoter (Talior-Volodarsky *et al.,* 2014). Based on these findings and on the results that implanted steers had higher carcass weight, which is reflective of higher muscle deposition than non-implanted steers reported in Chapter 2 of this thesis, it was expected that *ITGB1* and *ITGA11* in muscles from implanted steers in the current study would be upregulated. This is because increased collagen synthesis is required to enable the connective tissue to support the growing muscle. This was however not realized as these two integrins were downregulated in muscles from implanted steers. This result is however advantageous for meat quality as it shows that steers can be implanted to enhance yield without negatively affecting background toughness. A plausible explanation for this result is that steers treated with steroids grow faster and are therefore closer to their mature size at slaughter compared to steers not treated with steroids. In a study by Lee et al. (1990), steers untreated with TBA and E_2 had slower growth compared to untreated bulls during the growing phase. Thus, steers treated with steroids plateau in their growth in the finishing stage which may result in reduced collagen synthesis, hence the down-regulation of *ITGB1* and *ITGA11* found in the current study in implanted steers.

In a study by Sato et al. (1991), treatment of rabbit uterine cervical fibroblasts with steroids (progesterone or estradiol-17/1) decreased the steady-state RNA levels of pro-collagenase and pro-

stromelysin. On the other hand, steady-state *TIMP* mRNA levels were increased by treatment with the steroids. It was observed however, that progesterone exhibited more effect compared to estradiol-17/1. Contrary to the above-mentioned study, results from the current study showed an increase in the expression of the gene *TIMP2* in muscles from non-implanted steers. Although similar steroids were used in the above-mentioned study and in this thesis, a second (terminal) steroid composed of trenbolone acetate and estradiol was used in this thesis, which may be why the results obtained did not agree with those of Lee et al. (2007). Also, muscle obtained for gene expression analyses in this study were obtained after the payout period of the second implant. Thus, another plausible explanation is the additive effect of the second implant on the first implant. According to Fassina et al. (2000), TIMPs are involved in the regulation of other biological processes including cell growth, repression of angiogenesis, and the initiation or reduction of apoptosis. These activities may potentially influence meat quality. According to Ma et al. (2021), apoptosis has been proposed to be the first step involved in the process of muscle conversion to meat. Although the potential role of apoptosis in *post-mortem* proteolysis has been suggested, the inherent mechanisms through which the changes that occur in the metabolome in muscles affect processes involved in apoptosis and proteolysis, leading to variation in meat quality remain undetermined (Ma *et al*., 2021). Apoptosis has been suggested to be the first phase involved in the conversion of muscle to meat, with possible active interaction with *post-mortem* proteolysis (Ouali *et al.*, 2006). While TIMP1 is known to stimulate kinases involved in pathways for cell survival and proliferation (Li *et al.*, 1999), TIMP3 is known to increase apoptosis in muscle cells (Baker *et al.,* 1998). According to Fassina et al. (2000), the mRNA expression of TIMP2 is inhibited by growth factors in humans (Fassina *et al.,* 2000). This agrees with results from the current study where expression of *TIMP2* was downregulated in muscles from implanted steers. The downregulation of *TIMP2* in the current study in muscles from implanted steers is an indication of reduced inhibitory action of TIMPs against MMPs, which should result in an improvement in meat tenderness through a possible increase in collagen solubility at day 3 *post-mortem* as collagen would have more potential for degradation. The probable effect of the downregulation of *TIMP2* at day 3 *post-mortem* could possibly explain the increase in soluble collagen at day 12 *post-mortem,* as reported in Chapter 3. Results reported in Chapter 2 of this thesis however showed significant differences in the WBSF values of muscles from implanted and non-implanted steers, where muscles from implanted steers had a higher mean WBSF value. The effect of ageing on

meat tenderness, also reported in Chapter 2 of this thesis, showed reduced WBSF values in muscles after *post-mortem* ageing. This corroborates the suggested interaction between apoptosis, which is influenced by TIMPs particularly TIMP3, and *post-mortem* proteolysis, resulting in improved beef tenderness. The down-regulation of *TIMP2* in muscles from implanted steers suggests that collagen may not be responsible for increased toughness in meat from implanted steers. Further studies are warranted to establish the effect of hormonal steroids on TIMP mRNA abundance.

Similarly, the *CAST* gene was downregulated in muscles from implanted steers. Growth and carcass traits are regulated by many genes and are traits of economic importance in livestock. The selection of animals of higher growth rate and high quality carcass composition is very important to breeders, as well as consumers (Nickmard *et al.,* 2012). The rate of protein degradation in the muscle is key to the regulation of muscle mass. The differences observed in the rate of muscle growth in domestic animals are usually due to the differences in the rate of degradation of muscle protein, where limited or no change in the rate of protein synthesis occurs (Nickmard *et al.,* 2012). As steers treated with steroids had a higher growth rate than steers not treated with steroids (reported in Chapter 2 of this thesis), it is possible that *CAST* was downregulated in implanted steers to allow for their faster growth which eventually resulted in heavier carcasses from implanted steers compared to non-implanted steers. Additionally, the downregulation of *CAST* in muscles from implanted steers suggests that although these muscles had a higher mean WBSF value as reported in Chapter 2 of this study (also involved in an interaction effect with *post-mortem* ageing) compared to muscles from non-implanted steers, the activity of calpastatin may not be responsible for it. It cannot be explained at this stage why genes associated with collagen synthesis were highly expressed in non-implanted steers relative to implanted steers as the opposite of this result was rather expected. Further studies on the effect of steroids on the expression level these genes are therefore warranted.

Remodeling of the IMCT results in the synthesis of new collagen molecules (types I and III) and degradation of damaged or old collagen molecules (Karsdal *et al.*, 2016). MMPs degrade ECM compounds (Visse and Nagase, 2003), with their activity regulated at three levels (Manello and Medda, 2012). These include the transcriptional level where growth factors and cytokines trigger a signalling pathway leading to the activation of transcriptional factors, through activation via proteolysis, and through inhibition by TIMPs. Results from collagen heat-solubility reported in

Chapter 3 of this thesis revealed that while steroids interacted with RFI status to affect the total collagen concentration obtained during the PYR crosslink assay, steroids had no effect on the total collagen concentration obtained from EC crosslink assay. Furthermore, while RH supplementation actually increased the total collagen concentration obtained when measured using the PYR crosslink assay, it tended to increase the total collagen concentration obtained from EC crosslink assay. It was also revealed in the preceding chapter that both growth promotants used increased the density of PYR crosslinks at the collagen/molecular level (mol PYR/mol collagen) and in the raw meat (nmol PYR/g raw meat). For the crosslink EC however, while RH increased its density in the raw meat (nmol EC/g raw meat), steroids reduced its density at the collagen/molecular level (mol EC/ mol collagen), suggesting that PYR was preferentially synthesized when steroids were applied. These results support those of Roy et al (2015) and indicate that steroids and RH control collagen crosslink type through different mechanisms. The expression levels of *LOX, LHI* and *LH3* were neither affected by RFI status nor growth promotants. It is possible that other genes not profiled in the current study are responsible, such as LH2, particularly LH2b, the upregulation of which has been associated with increased PYR (Yamauchi and Sricholpech, 2012; Remst *et al.,* 2013); therefore, further studies on growth promotant effects on all possible genes implicated in collagen crosslink synthesis are warranted.

TGF-β1 is a growth factor known to regulate fibrogenesis (Lu and Pravia, 2010) through signalling activities involving the SMAD family that result in the expression of fibrogenic genes that catalyze the formation of collagen and collagen crosslink (Messague and Chen, 2000). *TGF-β1* has been found to activate down-stream SMAD signalling which consequently leads to the synthesis of collagen and collagen crosslinks (Letterio and Roberts, 1998) (Figure 4). TGF-β1 first binds to TGF-β receptor II (TβRII) which leads to the activation of TGF-β receptor I (TβRI). SMAD2 and SMAD3 (receptor-regulated SMADs or R-SMADs) are phosphorylated and bind to SMAD4 (Suwanabol *et al*., 2011), forming a SMAD complex which is translocated to the nucleus. The SMAD complex binds to binding elements of target genes that are specific to SMAD and activate the expression of fibrogenic genes which include procollagen and enzymes that catalyze the synthesis of collagen and crosslinks (Massague and Chen, 2000). *TGF-β1* mRNA expression levels obtained in this study were unaffected by RFI, steroids, or RH supplementation. This result indicated that it is unlikely that a pathway involving TGF-β1 was the mechanism responsible for increasing PYR density. On the other hand, following RH supplementation, the *β*-AA binds to *β*-

adrenergic receptors on the cell membrane and activates the *β*-adrenergic receptors, which is coupled to Gs proteins. This further activates adenylyl cyclase, leading to the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP is an intracellular signalling molecule which then binds to the regulatory unit of protein kinase A (PKA), leading to the release of its catalytic subunit. Phosphorylation by PKA results in the regulation of intracellular enzymes (Moody *et al.,* 2000). The intracellular enzymes include lipolytic and lipogenic enzymes involved in the synthesis of de novo fatty acids and triglycerides (Moody *et al.,* 2000). The binding action of *β*-adrenergic agonists to adrenergic receptors in adipose tissues leads to the activation of hormone-sensitive lipases and hydrolysis of triacylglycerols, and inactivation of lipogenic enzymes that are involved in the *de novo* synthesis of fatty acids and triglycerides (Mersmann, 1987; Yang and McElligott, 1987). This may potentially result in reduced fat synthesis. Lipogenic enzymes include fatty acid synthase and acetyl-CoA carboxylase (Bryant *et al*., 2010). A study by Miller et al. (1998) reported reduced activity of lipogenic enzymes in heifers administered the *β*-AA clenbuterol. Furthermore, in a study where adipocytes were isolated from Sprague-Dawley rats fed ractopamine, they were found to show not only an increase in lipolysis, but also an inhibition of lipogenesis (Hausmann *et al*., 1989). Further studies are however warranted to ascertain the effect of ractopamine on bovine lipolytic and lipogenic enzymes. Treatment of domestic animal species with *β*-AAs usually results in hypertrophy of the skeletal muscle possibly through the reduction of the degradation rate of myofibrillar protein (Bardsley *et al*., 1992). The breakdown of myofibrillar protein is associated with the proteinase system, which is calcium-dependent, constituting calpain and calpastatin, and whose activity is altered during *β*-AA administration (Bardsley *et al*., 1992). Generally, results from studies have supported the hypothesis that *β*-AAs enhances protein synthesis and/or inhibit protein degradation (Bergen *et al.,* 1989; Moloney *et al;* 1991). According to Smith et al. (1989), cattle treated with ractopamine showed an increase in the mRNA abundance of myosin. The expression and activity of enzymes involved in proteolytic degradation have been used to predict protein degradation rates. These include calpains, which are also intracellular enzymes, and their inhibitor, calpastatin (Goll *et al.,* 1998). According to Parr et al. (1992), an increase in the expression of calpastatin and a decrease in the expression of calpain implies a total decrease in the degradation rate of protein in cattle treated with the *β*-AA cimaterol. Regulation of the above-mentioned intracellular enzymes leads to intercellular signaling in the nucleus of the muscle which results in the increase in total mRNA

for myofibrillar proteins, reflected in an increase in the fractional rate of protein synthesis in the steers. This is accompanied by remodeling of the IMCT to accommodate the growing muscle, leading to the increase in total IMCT collagen concentration.

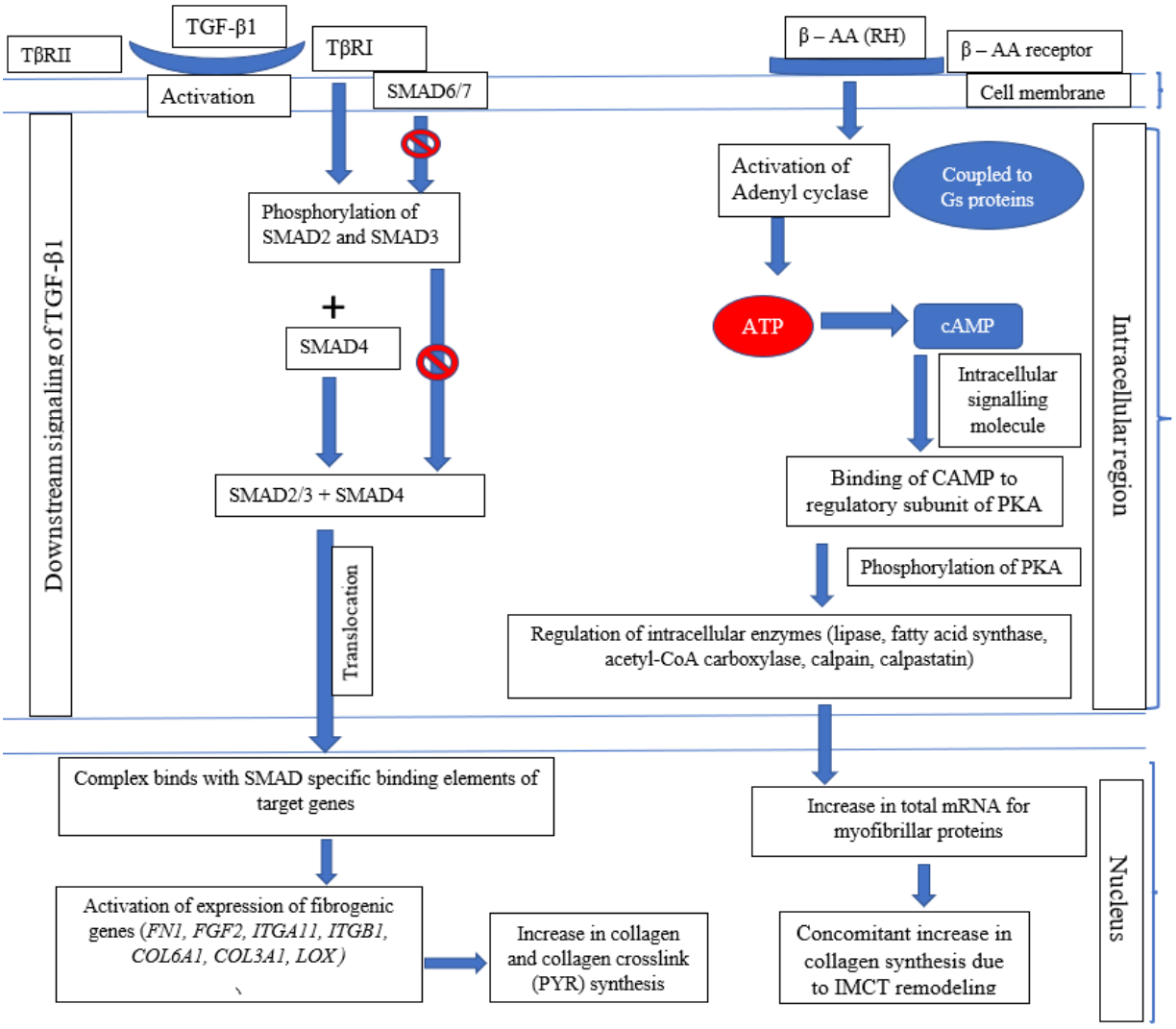

Figure 4.8 Schematic diagram representing the main pathways by which the growth factor *TGF-β1* and the *β-AA RH* influence the mRNA abundances for proteins associated with collagen and collagen crosslink synthesis.

(PKA – Protein kinase; TβRI – TGF-β receptor I; TβRII – TGF-β receptor II; ATP – Adenosine triphosphate; cAMP – Cyclic adenosine monophosphate; Gs protein – Guanine nucleotide-binding proteins)

Pearson's correlation analyses results showed relationships between *TGF-β1* and *FGF2, FGFR1*, *SMAD3, SMAD3, ITGB1*, where an increase in the expression level of *FGF2, FGFR1, SMAD2, SMAD3* and *ITGB1* was associated with an increase in *TGF-β1. FGFR1* was also strongly co-expressed with *ITGA1* and *ITGA11.* Fibroblast growth factors (FGFs) are signalling proteins that are secreted and possess a wide range of function including cell proliferation and development, as well as wound healing (Mohammadi *et al.,* 2005). As detailed earlier in this thesis, these include the regulation of cell growth and differentiation, as well as the expression of collagen (Florini *et al.,* 1986; Roberts *et al.,* 1986). Expressed primarily in endothelial cells, fibroblasts, hematopoietic cells, as well as smooth muscle cells, *TGF-β1* activates down-stream *SMAD* signalling, which regulates the expression of collagen in a steady state (Schnabl *et al.,* 2001). As such, the co-expression of *TGF-β1* with *FGF2, FGFR1, SMAD2,SMAD3* and *ITGB1* (which are all collagen synthesising genes) supports the function of fibronectins and integrins in collagen synthesis. The *LOX* gene, as stated above, is involved in the synthesis of collagen crosslinks through oxidation of lysine residues in the peptide region of collagen (Kagan and Li, 2006). The mRNA expression of *LOX* earlier explained may possibly be controlled by *TGF-β1* (Uzel *et al.,* 2001). This justifies the strong co-expression of *LOX* and *FGF2*, and *LOX* and *ITGB1*, as crosslinks are required to join collagen molecules synthesized by *LOX* and *ITGB1* together. A balance is therefore required between these two groups of genes in the regulation of the concentration of collagen and the density of associated crosslinks (Dhalla *et al.,* 1996).

Remodelling of the IMCT facilitates the synthesis and deposition of new collagen molecules. As such, the co-expression of *MMP2* and *MMP8,* with TIMP1, found in this study was expected and is justified. *LH1* was co-expressed with *FGF* and *TGF-β1. LH1* is particularly responsible for the hydroxylation of lysine residues in the helical regions of both fibrillar and non-fibrillar collagens (Kivirikko, 1982). It is therefore expected that as collagen deposition increases, the synthesis of collagen crosslinks also increases to allow for the arrangement of the collagen molecules. The gene *P4HA1* was co-expressed with *SMAD2, MMP2, MMP8.* Interestingly, *P4HA1* was co-expressed with *TIMP1* but not with *TIMP2.* According to Myllyharju (2003), *P4HA1* controls the formation of proper folding of newly synthesised chains of procollagen. As signalling from *SMAD2* leads to the synthesis of new collagen, *P4HA1* is required to ensure the proper folding of the procollagen. This justifies its co-expression with *SMAD2*. Furthermore, to

ensure a balance in ECM synthesis and degradation, both MMPs and TIMPs are required. As expected, *MMP2, MMP8* and *TIMP1* were co-expressed with *P4HA1.*

For correlations between the expression levels of target genes profiled and growth performance characteristics, most of the significant relationships were between target genes, and weight parameters, feed intake and feed conversion efficiency. Unexpectedly, no significant correlation was found between growth hormone genes such as *IGF-1* and *TGF-β1*, and growth characteristics. According to a study by Xu et al. (2014), *FGFR1*, from co-expression analysis, was reported to be a gene related to weight in the skeletal muscle of ovine during the stages of development. Furthermore, Sun et al. (2015) reported significant differences in mRNA expression levels of *FGFR1* in the skeletal muscle of Qinchuan cattle, during three different developmental stages. The inverse correlation between *FGFR1* and initial weight before steroid treatment found in this study was therefore unexpected. In a study by Qi et al. (2015), the expression of *MMP8* in pregnant cows was found to decrease significantly from 135 days *pre-natal* to 30 months *post-natal.* According to the said study, *MMP8* expression level is minimized greatly during pregnancy to 30 months *post-natal*. This may explain why *MMP8* expression level in this study was inversely related with initial weight before steroid treatment, as steers were about 11 months of age before they were treated with steroids. The negative relationship between *ITGA1* and *COL3A1* was unexpected. This is because *ITGA1* has been shown to bind to several collagen types including collagen type III (COL3A1) (Gardner, 2014) to result in further synthesis of collagen. *ITGA1* and *COL3A1* were therefore expected to be co-expressed. The negative correlation between *ITGA1* and *COL3A1* cannot be explained at this point.

4.6 Conclusions

This study confirmed that selection for divergent RFI in a single generation potentially results in differences calpastatin (*CAST)* gene expression levels, as low RFI steers showed a tendency toward increased expression of *CAST.* As a result of the increased level of expression of the *CAST* gene in low RFI steers in this study, selection for low RFI animals (steers) should be implemented with caution as it may no longer be economically viable when tougher meat is produced. Furthermore, the study showed that low RFI steers, when treated with the two growth promotants used in the current study had reduced expression of *CAPN1,* which is needed for *post-mortem* muscle proteolysis. This is an indication of the additive effect of growth promotant of

CAPN1 expression. This study also showed the tendency for *MMP13* to be downregulated in low RFI steers, which may ultimately result in less tender meat as *MMP13* is an important collagenase required for collagen degradation. Treatment with steroids downregulated the expression levels of genes associated with proteins involved in collagen synthesis such as *ITGB1*, *ITGA11, TIMP2* and *FN1*. That steroid utilization had some main effects on the expression levels of some genes, while supplementation with ractopamine hydrochloride had no main treatment effect nor tendencies on any of the genes expressed suggests different mechanisms through which these two growth promotants work, and further influence gene expression levels. Results from this study did not show the growth hormone genes *IGF-1* and *TGF-β1* to be responsible for the efficient production performance in implanted cattle. Further studies are however required to ascertain the effect of gene expression levels on growth performance and feed conversion efficiency in cattle. The target gene *SMAD2* was observed to be the most important collagen synthesising gene among the R-SMAD family in the *semimembranosus* muscle in this study, due to its consistent high expression level regardless of treatment.

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Chapter 5. General Summary, conclusions, and recommendations for further research

5.1 Overview

The implementation of selecting for RFI to improve feed efficiency has been well documented (Nascimento *et al*., 2016). Growth promotants have also been incorporated into beef production systems to improve efficiency, and increase carcass yields (Ebarb *et al.,* 2017). According to Duckett and Pratt (2014), anabolic implants and β-adrenergic agonists (β-AA) are the two commonest growth promotants used by producers. Duckett and Pratt (2014) reported an improvement in feedlot performance and carcass characteristics, and the generation of a profit of $163 per head, when implants containing trenbolone acetate (TBA) and estradiol (E2) were used. Although the use of growth promotants have been reported to increase beef toughness, studies have shown that meat tenderness can be improved by *post-mortem* ageing (Judge and Aberle, 1982; Etherington, 1987, Ebarb *et al*, 2017). According to Stanton and Light (1988, 1990) however, collagen found in the perimysial region of the IMCT is deformed and solubilized to some extent during *post-mortem* ageing. To produce meat of high quality however, it is imperative to assess variables associated with animal production. These include genetics and management, which are associated with the regulation of muscle gene expression (Hocquette *et al.,* 2007).

Although the use of the aforementioned growth promotants result in similar advantages in animal production, they differ in their utilization and mode of action (Johnson *et al*., 2013). In the current study, hormonal steroids were given as implants while RH was given as a feed additive. The mechanisms through which these growth promotants work have been the subject of many studies but largely remains inconclusive. IGF-1 has been reported to increase in circulation when steroid implants are used in meat producing animals such as steers (Johnson *et al.,* 1996). IGF-1 is also a known effective growth factor that stimulates the growth and differentiation of the skeletal muscle by stimulating protein synthesis while reducing protein degradation in the skeletal muscle. According to Johnson et al. (2013), the use of combined TBA/E_2 implants increase carcass protein by about 10%. The combined implant TBA/E_2 was the second and terminal implant used in the current study after an initial combined implant made of progesterone and estrogen, and as such, its sole and direct effect on protein synthesis could not be assessed. According to Johnson et al. (2013), the increase in protein synthesis in implanted steers occurs during the first 40 days after

animals are implanted. Implants that contain anabolic steroids have been reported to cause hypertrophy in postnatal skeletal muscle, which has been partly attributed to an increase in circulatory as well as skeletal muscle-produced IGF-1 (Johnson *et al*., 1996; Johnson *et al*., 2013). According to Cronje (2002) and Barkawi et al. (2009), the increase in circulatory IGF-1 is related with an increase in growth rate in cattle. In the skeletal muscle, IGF-1 has been found to stimulate the uptake of amino acids, inhibit proteolysis (Shimizu *et al*., 1986; Fryburg, 1994) and enhance the efficiency of utilization of feed (Yilmaz *et al.,* 2004). The administration of the implant TBA/E_2 in steers has been reported to increase the number of satellite cells that are actively proliferating within an implantation period of 35 days, the effect of which is possibly mediated by IGF-1 produced by the skeletal muscle. The increase in the proliferative activity of the satellite is likely to improve the growth rate of muscle in cattle. Overall, the above explanation supports the mechanism where muscle growth in beef cattle is induced by steroid implants that results in an increase in local production of IGF-1 in the skeletal muscle. This will lead to the increase in the activity of satellite cells, resulting in increased amounts of DNA in the muscle, and subsequently increase growth in the skeletal muscle (Johnson *et al*., 2013).

β-AAs are known to have a repartitioning activity due to their ability to cause the use of free fatty acids as opposed to amino acids in animals when they are supplemented with it (Ricks *et al*., 1984), resulting in muscle hypertrophy and reduce protein degradation. Although the most reported effect of β-AA supplementation is the increase in the mass of skeletal muscle, many of the studies reported either no change or a decrease in the amount of DNA because of β-AA supplementation (O'Conner *et al.,* 1991; Johnson *et al*., 2013). Researchers have now focused their attention on the direct binding of β-AA to their receptors, which either affects the rate of synthesis or degradation of protein, or both. β-AA receptors are abundant on the cell surface of the skeletal muscle. The net effect of the changes in the increase in protein synthesis and decrease in protein degradation result in striking changes in the accumulation of protein in the skeletal muscle. It would seem that β-AA incite efficiency of nuclei that exist in the myofibre in order to increase the accumulation of muscle protein without the need for additional DNA from satellite cells (Johnson *et al*., 2013).

5.1.1 Relationships between residual feed intake, the use of growth promotants, and the growth and meat quality characteristics of crossbred Angus steers

This study was unique in the sense that it compared growth performance, carcass, and meat quality characteristics among steers of low and high RFI status treated with hormonal implants and/or RH with additional focus on how *post-mortem* ageing influences beef tenderness. The results obtained from this study indicated a potential for hormonal implants to interact with low RFI to increase ADG, and for hormonal implants to increase slaughter and carcass weights, as well as beef toughness (Dikeman *et al.*, 1999; Ebarb *et al.,* 2017). Although similar circulatory levels of IGF-1 were found among implanted and non-implanted steers, results indicated that implanted steers consumed more feed than non-implanted steers, with an increased ADG compared to non-implanted steers. This study also validated findings from literature that low RFI animals and implanted animals are more feed efficient compared to high RFI animals and non-implanted animals (Nichols *et al.,* 2002; Nkrumah *et al.,* 2004). For beef producers, selection for low RFI and the use of hormonal implants can be beneficial, and instrumental in the reduction of feed costs, to increase profitability in beef production. Although the increase in the HCW in the current study was not attributed to an increase in circulatory IGF-1 due to steroid implant alone, it can still be argued that IGF-1 was responsible, as implanted steers in the current study obtained higher hot carcass weight (HCW) compared to non-implanted steers. It can be argued that the reason for the lack of steroid treatment effect on circulatory IGF-1 is due to insufficient sampling to accurately quantify circulatory IGF-1.

Results from the first chapter also validated literature findings on the reduction in beef tenderness due to the use of hormonal implants, and the use of animals selected for low RFI. This was observed in the variation in beef tenderness in SM muscles from implanted and non-implanted steers, and from low and high RFI steers, and validates findings from the study by literature (Nascimento *et al.,* 2016). Producers can however choose increased ageing periods for beef obtained from low RFI cattle, to obtain desired tenderness in their beef cuts. Alternatively, certain processing technologies can be employed on beef cuts obtained from low RFI cattle to enhance tenderness. Food ingredients including sodium tripolyphosphate and sodium chloride can bind water and enhance the solubility of protein. Injecting cuts of beef that are less tender with sodium tripolyphosphate and sodium chloride has been shown to improve tenderness (Hoffman, 2006;

Holmer *et al.,* 2009). The blade tenderization technique, another processing technique described by Kolle et al. (2004) which was successful in reducing WBSF values of the caudal portion of the *m. semimembranosus* from 51.5 N to 42.9 N. The incorporation of such techniques during processing will potentially result in increased profit for the producer.

Results from this chapter also highlighted *post-mortem* ageing to have the most effect on meat quality characteristics, particularly, on beef tenderness as measured by the Warner-Bratzler Shear Force (WBSF) and corroborates findings from Ebarb et al. (2017). The results revealed the potential for *post-mortem* ageing to remove the negative effect of growth promotant use on beef tenderness, all of which are immensely beneficial to the cattle and beef industry. We therefore accept the hypothesis that growth promotants, particularly hormonal implants increase growth performance characteristics which increases profitability for producers, but unfortunately also increases beef toughness which may lead to economic losses. This can however be prevented or overcome with *post-mortem* ageing.

Based on the results from this study, we reject the hypothesis that growth promotants increase steer growth performance characteristics through an increase in the circulating levels of IGF-1. Blood sampling for circulating IGF-1 and IGFBP-3 quantification in the first experimental study occurred 30 days after the second implantation, where the effect of the hormonal implants on IGF-1 concentration possibly would not be accurately detected. For future studies, and to accurately measure circulating IGF-1 and IGFBP-3 levels, blood samples must be taken on the day before implants are administered (basal samples), and serially afterwards as described in the study by Johnson et al. (1996a). In the said study, the treatment of crossbred steers with a combined implant containing trenbolone acetate and estradiol (TBA/E_2) resulted in an increase in circulating IGF-1 concentration. According to Pampusch et al. (2003), hormonal implants including trenbolone acetate with estrogen administer their effect through IGF-1. In a study by Johnson et al. (1996b), steers implanted with TBA/E_2 showed significant increase in the deposition of carcass protein. This increase in the deposition of carcass protein coincided with the period during which circulating IGF-1 concentration was elevated in the study by Johnson et al. (1996a). These results suggest that the increase in circulating IGF-1 concentration due to hormone implant administration could play a role in enhancing the deposition of carcass protein, hence muscle hypertrophy in feedlot cattle. Overall, results from the first study (Chapter 2) stressed on the potential of hormonal growth promotants to increase production efficiency including feed conversion efficiency, ADG,

slaughter and carcass weight, and reduce beef tenderness. Furthermore, the results highlighted the importance of *post-mortem* ageing in resolving beef toughness due to implant utilization. We therefore accept the hypothesis that while growth promoting technologies, particularly steroid implants enhance feedlot performance and carcass gain, they also reduce beef tenderness. The negative effect of steroid implants on tenderness was however removed by *post-mortem* ageing.

5.1.2 Effect of Residual Feed Intake (RFI) and Hormonal Growth Promotants on collagen characteristics and their role in tenderness of the *semimembranosus* and *gluteus medius* muscles

This study was executed to analyze the effect of growth promotants and RFI status on collagen solubility measures in the GM and SM muscles, and collagen crosslink densities in the SM muscle. Results from this experimental study indicated the potential of hormonal growth implants to reduce the insoluble collagen content of the GM muscle. These results support the theory that new soluble collagen is deposited in the network of connective tissues during muscle development (Purslow, 2014). This would potentially lead to a reduction in the insoluble collagen portion of the total collagen concentration as was observed in this study. Results for the SM muscle suggested the potential for *post-mortem* ageing to interact with steroids and RH individually, and with RFI status to increase solubility.

Results also highlighted the potential of *post-mortem* ageing to increase collagen solubility. Days of *post-mortem* ageing had the most significant effect on the measures of collagen heat-solubility in both GM and SM muscles in this study, particularly on the soluble collagen content, and hence, solubility percentage. This validates findings from literature that disprove the long-standing notion that the IMCT was unchangeable during *post-mortem* ageing of meat (Ebarb *et al*., 2017). This was reflected in both muscles used in this study as steaks aged for a longer period had reduced WBSF values, as reported in Chapter 2 of this thesis. Regardless of treatment used in the study, collagen solubility percentage of the GM muscle increased with days of *post-mortem* ageing. In the SM muscle however, the increase in the percentage of collagen solubility was observed in meat from non-implanted steers unselected for low RFI, as well as in meat from implanted, low RFI steers. These results support findings of degradation of the IMCT during *post-mortem* ageing, and ultimately confirms that collagen is subjected to proteolysis during *post-mortem* ageing by the activity of enzymes that most likely belong to the family of matrix metalloproteinases (Wu *et al.,* 1991; Knauper *et al*., 1996; Sylvestre *et al.,* 2002).

In addition to the determination of the densities of the crosslinks PYR and EC in the SM muscle, the second experimental study (Chapter 3) also focused on the analyses of their associated collagen content in the perimysium. Results from this study on the associated collagen content in the perimysium determined by the PYR assay suggest a tendency for intramuscular collagen

content to differ between high and low RFI steers due to the application of steroids, and not RFI status alone. The results further validated that implantation with hormonal steroids, which contained a synthetic testosterone analog, increased the density of PYR in the SM while it decreased the density of EC, and validates findings from literature that PYR is the major trivalent crosslinks associated with beef toughness (Roy *et al.,* 2021), and potentially results in reduced collagen heat solubility. This was further validated in the study by a positive correlation between PYR and WBSF. Furthermore, the results showed that supplementation of steers with RH increased the concentrations of collagen from PYR crosslink assay, with a potential of increasing that from EC crosslink assay.

RH supplementation also resulted in increased PYR and EC crosslinks, which is an indication that RH increased the synthesis of new collagen molecules, as well as deposition of the two mature or trivalent crosslinks, which validates literature findings (Girard *et al.,* 2011; Roy *et al.,* 2015). To alleviate the effect of both growth promotants on collagen and collagen crosslink synthesis, reduced amounts can be used. Additionally, increased *post-mortem* ageing of muscles from animals treated with growth promotants can implemented to address the contribution of collagen to beef toughness. This way, profits will be made from increased muscle deposition as a result of the use of the growth promotants, as well as from good tender meat due to *post-mortem* ageing. Results from this study indicated that RFI status and RH supplementation had no effect on collagen solubility measures in both GM and SM muscles which validates findings from literature (Girard *et al.*, 2011; Fidelis *et al.,* 2017). We accept the hypothesis that growth promotants increase density of the mature crosslinks. Additionally, we accept the hypothesis that *post-mortem* ageing increases collagen solubility measures by weakening the structure of the IMCT.

5.1.3 Profiling of the expression of genes related to connective tissue synthesis and degradation as affected by RFI status and growth promotant use and their relationship to beef quality

The third experimental study (Chapter 4) focused on the expression of genes that are associated with beef toughness, mainly contributed by the IMCT from SM muscle samples. In this study, the main objective was to determine the effect of RFI status and growth promotant use on the level of expression of genes associated with collagen and collagen crosslink synthesis. We

hypothesized that the use of growth promotants increases the level of expression of collagen and collagen crosslink synthesizing genes. Additionally, we hypothesized that selection for RFI results in high expression levels of genes associated with collagen crosslink synthesis, and also increase the gene expression level of *CAST,* while reducing the expression level of *CAPN1.* This is the first study that comprehensively focuses on the effect of selection for RFI and use of growth promotants on the expression levels of genes associated with beef tenderness, particularly collagen synthesis.

Results from the third experimental chapter showed that *CAPN1* expression level was reduced in muscles from steers selected for low RFI only when treated with steroids and RH, which is an indication of a difference in the response to growth promotants between low and high RFI animals. Results from this experimental study further showed the tendency for low RFI steers to have increased mRNA expression of the *CAST* gene, validating results from literature (McDonagh *et al.*, 2001). These results contribute to the validation of the result of different WBSF values found among muscles from low and high RFI steers in the first experimental study (Chapter 2). This further indicates that the different WBSF values, hence different levels of beef tenderness, may be due to different levels of myofibril degradation because of different mRNA levels of *CAST*. These results are especially important for producers as the expression levels of *CAPN1* and *CAST* is crucial for beef tenderness, which influences profitability for producers. This result can also be used as a deciding factor on the treatment of cattle selected for improved RFI with growth promotants. Results from the current study on IGF-1 expression did not validate that the use of steroids tended to increase IGF-1 expression level, while RH decreased it (Walker *et al*., 2007). The results from this study on IGF-1 expression level however validates results reported in the first experimental study (Chapter 2) where circulating IGF-1 levels were unaffected by neither RFI nor growth promotants.

Steroids unexpectedly decreased the mRNA expression levels of *ITGA11, ITGB1, TIMP2,* and *CAST*. It was expected that *ITGA11* and *ITGB1* would be upregulated to induce the synthesis of new collagen in order to support the increased growth, and hence the growing frame of implanted steers. As this was not realized in this study, we reject the hypothesis that steroid implants increase the expression levels of genes associated with collagen and collagen crosslink synthesis. Furthermore, although utilization of the two growth promotants in this study had varying effects on the densities of mature crosslinks PYR and EC reported in Chapter 3 of this thesis,

neither growth promotant influenced the level of expression of any of the crosslink synthesising genes (*LOX, LHI* and *LH3*) expressed in this study which does not support the results on PYR and EC in Chapter 3. It is worth noting however, that *LOX* was similarly and consistently moderately expressed among the treatment groups and can be implicated in the expression of PYR, which is the major stable crosslink associated with beef toughness compared to EC (Bosselmann *et al.,* 1995; Roy *et al.,* 2021), due to its heat and acid resistance (McCormick, 1994) which was validated in Chapter 3 of this thesis and earlier explained in this chapter. Further research is warranted to study the effect of steroids on collagen synthesizing genes for more clarity and understanding. That RH had no effect on the gene expression level of all 31 genes while steroid implant influenced the expression levels of some shows that the two growth promotants used in the study have different modes of mechanisms, and effects on mRNA expression of genes. Alternatively, it could also be as a result of lesser amount of RH consumed by steers. Although the crosslinks formed because of steroid implant treatment remained well after the pay-out period, muscle sampling for gene expression was carried out at the end of the study (RH treatment). To obtain accurate results that better reflect the effect of steroid implants and RH on the expression of genes associated with collagen and collagen synthesis, it is recommended that biopsies be taken before, during and after steroid implant and RH treatment.

5.2 Gaps and future recommendations

On the whole, this study touched on the major beef production stages up until slaughter, which have presented some major economic challenges to producers. In as much as the best effort was put into having a thoroughly comprehensive study, the authors acknowledge that some experimental procedures could be done differently to improve upon the study. At stated earlier, one crucial thing that could be done differently is the blood sampling for the quantification of circulating IGF-1 and IGFBP3. Ideally, one blood sampling should be done before treating cattle with steroid implants. This would serve as a control to accurately report on how much increase in IGF-1 and IGFBP3 resulted due to steroid implant use. Additionally, several sampling can be done over the pay-out period of the implants to ascertain if their peak concentration period coincides with their peak growth period, and also when their concentrations begin to dip and plateau. This will provide accurate information on actual pay-out periods and when the second and/or terminal implant can be administered. An example of how this serial sampling can be done is seen in the

study by Johnson et al. (1996a). Other methods of quantification, such as the radioimmunoassay method can also be used in future quantification of circulating growth factors, in addition to the ELISA method. Results from the two methods can be compared to accurately determine growth factor concentration due to steroid implant use.

In order to ensure that experimental animals consume the desired amount of RH, accurate measures on actual feed intake should be done to facilitate accurate calculations for the incorporation of the β-AA into the diet of experimental animals. This will ensure that animals actually consume the expected amount of RH, in order to obtain the desired effect of increased muscle mass (Gruber *et al*., 2007). The finding that ageing for a 12 day period resulted in more tender beef compared to ageing for 3 days suggests proteolytic action of enzymes, and a possible weakening of the connective tissue, hence collagen structure. Although the ageing period of 12 days was effective in reducing WBSF values which denote increased beef tenderness, increased ageing periods can be implemented by producers. The number of experimental animals should also be increased to increase the reliability of results obtained. For all stakeholders in the industry particularly for producers, the timely and efficient use of hormonal growth promotants will go a long way in increasing profitability as more muscle of desired tenderness will be realized.

Although some studies have reported that increased muscle cross-sectional area and increased calpastatin activity are responsible for the associated toughness of muscles from implanted animals (Gerken *et al.,* 1995; Ebarb *et al.,* 2017), further studies into the exact causative mechanisms are warranted. This could further provide an insight into the periods within which they occur and aid in the ascertaining of the appropriate periods in the production system when these hormonal growth promotants can be utilized, without the adverse effect on meat tenderness. As reported by Boehm et al. (1998), the hormone testosterone enhances calpastatin activity, which reduces meat tenderness. Thus, to attain meat with desired tenderness, it would appropriate to implant cattle with hormones containing a testosterone analog at a time when the pay-out period would be far exhausted before the cattle are slaughtered. This could potentially reduce the calpastatin levels at slaughter, and result in beef of desired tenderness. Furthermore, the implementation of different feeding regimens and increased ageing period will potentially result in increased beef tenderness, which will enable producers to receive premiums for their beef cuts. The use of steers selected for low RFI and treated with hormonal steroids will also reduce the cost of feed which will invariably increase their profits, as feed intake will be reduced and feed conversion efficiency, increased.

Additionally, the use of cattle selected for low RFI has positive impact on the environment, as observed in the study by Fitzsimons et al. (2013) where results implicated that enhanced selection for enhanced RFI reduces methane emissions without any effect on the production performance of growing beef cattle.

A number of studies have reported that treating cattle with RH results in a decrease in muscle DNA concentration (Beermann *et al.,* 1987; Kim *et al*., 1987; O'Conner *et al.,* 1991). Although studies have reported an increase in muscle fibre diameter, particularly of type II muscle fibre type and subsequent hypertrophy due to supplementation with β-AA (Kim *et al.,* 1987; Miller *et al*., 1988; Smith *et al.,* 1995), it is recommended that total DNA concentration be determined in future studies in relation to treatment with β-AA. This will aid in ascertaining and understanding the actual source of DNA required for muscle hypertrophy, as treatment with steroid implants has been reported to provide the required DNA that subsequently facilitates muscle hypertrophy due to RH supplementation (detailed in Chapter 4). This will inform industry stakeholders particularly producers, about accurate timing for β-AA supplementation, especially after steroid implants have been used. The expression levels of other genes associated with meat quality, particularly meat tenderness, such as the peroxisome proliferator-activated receptor gamma (*PPARG*), CCAAT/enhancer binding protein alpha (*CEBPA*) and retinoid X receptor alpha (*RXRA*) which are known nuclear transcription factors recognized as major molecules involved in the regulation of adipogenesis (Goszczynski *et al*., 2016), *COL1A1, COL3A1* (Gonzalez *et al.,* 2014), and genes of specific heat-shock proteins (Bernard *et al.,* 2006) should also be profiled from muscle biopsies before and after treatment with growth promotants. This will provide information that will be beneficial to cattle breeders in improving beef tenderness. According to Blanco et al. (2013), the characteristics of collagen are not only different among the various muscles, but also different among breeds as a result of variation in the maturity rates of the breeds. Thus, different breeds can also be included in future studies to ascertain the effect of RFI status and growth promotant use on cattle growth performance, carcass and meat quality characteristics, and more critically, on the mRNA abundance of genes associated with collagen and collagen crosslink synthesis. In addition to gene expression, proteomics can be performed to validate the proteins of the expressed genes.

Overall, the study has been scientifically relevant in bridging the gap between how the use of growth promotants, RFI status, and their effects individually and through interactions, influence production performance, carcass and meat quality characteristics, and also through the expression

of genes that are directly associated with meat toughness. Results from this study will ultimately add to prior knowledge on growth promotants and their effects on cattle growth performance and meat quality characteristics. This study has further highlighted information on major factors that contribute to the inconsistencies in beef tenderness and has also suggested methods through which beef tenderness can be improved. Overall, results from this study will go a long way to advise on appropriate selection for residual feed intake, and on decisions involving the incorporation of growth promotants in steer production, for the production of high-quality beef, especially one that is consistently tender. Furthermore, this study will add on to previous knowledge on more efficient and sustainable methods of beef cattle production.

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